Supporting Information

Synthesis and Initial Pharmacology of Dual-Targeting Ligands for Putative Complexes of Integrin αVβ3 and PAR2

Mark W. Majewski,[†] Disha M. Gandhi,[†] Trudy Holyst,[‡] Zhengli Wang,[‡] Irene Hernandez,[‡] Ricardo Rosas, Jr.,[†] Jieqing Zhu,[‡,Δ] Hartmut Weiler,[‡,§] and Chris Dockendorff*[,†]

[†]Department of Chemistry, Marquette University, P.O. Box 1881, Milwaukee, WI, 53201-1881, USA

[‡]Blood Research Institute, Versiti, Milwaukee, WI, 53226, USA

[§]Department of Physiology, Medical College of Wisconsin, Milwaukee, WI, 53226, USA

[Δ]Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, 53226, USA

* Email: christopher.dockendorff@mu.edu Tel: +1-414-288-1617.

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1. Abbreviations

AcOH acetic acid
Arg arginine
conc. concentrated
Boc *tert*-butoxycarbonyl (protecting group)
BSA bovine serum albumin
cRGD cyclic RGD-containing peptide, aka cilengitide
CuAAC copper-catalyzed alkyne–azide cycloaddition
DCE 1,2-dichloroethane (solvent)
DCM dichloromethane (solvent)
DMAP 4-dimethylaminopyridine (nucleophilic catalyst)
DMF *N,N*-dimethylformamide (solvent)
DMSO dimethylsulfoxide (solvent)
EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (amide coupling reagent)
DAPI 4′,6-diamidino-2-phenylindole
DIPEA *N,N*-diisopropylethylamine (base)
Dmcp dimethylcyclopropylmethyl (protecting group)
DMEM Dulbecco's modified Eagle media
DODT 3,6-dioxa-1,8-octanedithiol
EtOAc ethyl acetate (solvent)
FBS fetal bovine serum
Fmoc fluorenylmethyloxycarbonyl (protecting group)
Fn fibronectin
GFP green fluorescent protein
GPCR G-protein coupled receptor
HATU (amide coupling reagent)
HBSS Hank's balanced salt solution
HEK human embryonic kidney
HEPES 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (buffering agent)
HUVEC human umbilical vein endothelial cells
HOBt 1-hydroxybenzotriazole (additive for amide coupling)
HPLC high performance liquid chromatography
IPA isopropyl alcohol
LCMS liquid chromatography-mass spectrometry
MeCN acetonitrile (solvent)
MFI mean fluorescence intensity
NMP *N*-methyl-2-pyrrolidinone (solvent)
NMR nuclear magnetic resonance
Orn ornithine
PAR protease-activated receptor
Pbf 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (protecting group)
Pd/C palladium on carbon
Phe phenylalanine
PTFE polytetrafluoroethylene
RBF round-bottomed flask
SEM standard error of the mean
Ser serine
TBTA tris(benzyltriazolylmethyl)amine
TFA trifluoroacetic acid
TFE 2,2,2-trifluoroethanol
THF tetrahydrofuran (solvent)
TIS triisopropylsilane
Vn vitronectin

2. Additional Assay Data

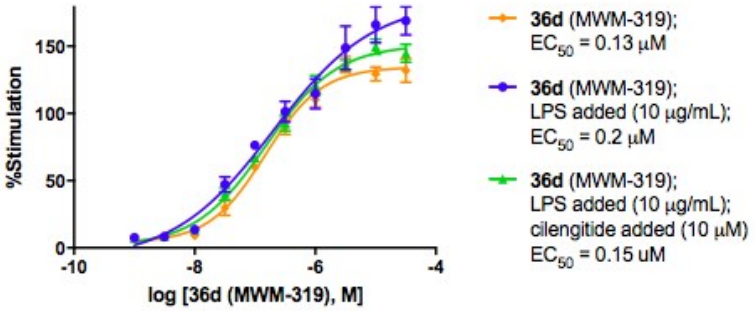


Figure S1. Stimulation of calcium mobilization in EA.hy926 cells pretreated with LPS by bivalent PAR2 agonist **36d** (MWM-319), with and without cilengitide as a competing ligand 30 min. prior to agonist addition. Cells were pretreated with 10 μg/mL LPS for 2 h prior to addition of agonist.

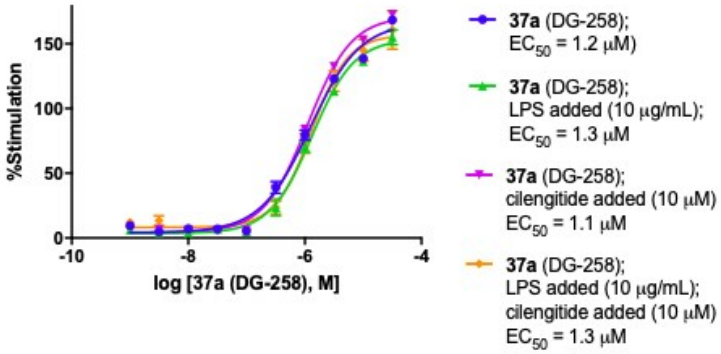


Figure S2. Stimulation of calcium mobilization in EA.hy926 cells pretreated with LPS by bivalent PAR2 agonist **37a** (DG-258), with and without cilengitide as a competing ligand 30 min. prior to agonist addition. Cells were pretreated with 10 μg/mL LPS for 2 h prior to addition of agonist.

3. Assay Protocols

a) General details

Water for solutions was deionized and filtered through charcoal (Milli-Q by Millipore) to a resistance of 18 MΩ. SLIGKV-NH_2 (TFA salt) (PAR2 agonist) was obtained from Bachem (cat# H-5042.0025) or AnaSpec (cat# AS-60217-1). 2f-LIGRLO-NH_2 (PAR2 agonist) was obtained from AnaSpec (cat# AS-65490). Cilengitide (TFA salt) was obtained from Millipore-Sigma (cat# SML1594).

Adherent EA.hy926 cells (ATCC, CRL-2922) were used for indicated assays. Cells were cultured using the suggested protocol from the manufacturer (ATCC), except that 150 cm^2 tissue culture flasks were used, and DMEM complete media was prepared as described below. EA.hy926 cells were frozen in 40% fetal bovine serum (FBS), 50% complete media, and 10% DMSO in liquid nitrogen, and used between passages 7 and 9. HUVEC were cultured as indicated below. All manipulations prior to the assays were performed in a sterile laminar flow cell culture hood.

Cell counting was performed with an automated cell counter (Countess, Invitrogen) using Invitrogen cell counting chamber slides and trypan blue stain. All assays were run in 96-well plates (Corning Costar #3603, polystyrene black wall, clear bottom). Unless otherwise noted, media exchanges with 96-well plates were performed using an automated liquid handler (Beckmann-Coulter, Biomek 3000) with 220 µL pipette tips. Cells were imaged with an EVOS Fl inverted microscope. All assays were run on a multimode plate reader (Perkin Elmer EnSpire). Data were exported to Microsoft Excel for nominal processing (calculation of normalized ΔRFU/basal levels), then analyzed with GraphPad Prism (versions 5 or 6) using 4-variable non-linear regression for concentration-response curve fitting.[1]

b) Culturing of HUVEC

Pooled primary HUVEC were purchased from ATCC (cat# CC-2519) and passaged according to the manufacturer's directions. Cells were frozen after passage #4, and these were used for all assays.

c) Preparation of complete media for EA.hy926 cells

Complete cell media (for EA.hy926 cells). Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose (500 mL) was supplemented with 200 mM L-glutamine (10 mL, 4 mM final conc.), 100 mM sodium pyruvate (2.5 mL, 1 mM final conc.), 7.5% (w/v) sodium bicarbonate (5.0 mL, 0.75 g/L final conc.), 100X penicillin and streptomycin (Corning #30-002-CI, 2.0 mL) and heat inactivated FBS (50 mL). The prepared media was stored in a refrigerator (5 °C) until needed.

d) Preparation of complete media for HUVEC

Endothelial Cell Growth Medium 2 (from Promocell; 500 mL; Fisher cat# 50-306-189). Before use, the bottle is treated with a Cell Media Supplement (from PromoCell and included with media purchase, 12.6mL; 1 tube is sufficient for the supplementation of a 500 mL bottle of media) and penicillin/streptomycin (100X, 2.0 mL).

e) Protocols for calcium mobilization assays

All calcium mobilization assays were run according to our previously published protocol using EA.hy926 cells.[1] For assays with HUVEC, **10,000 cells/well** were added to sterile 96 well Corning CellBind plates, black polystyrene with clear bottoms (Fisher cat# 07-201-96). The plates was incubated for 40 h at 37 °C with 5% CO_2. A media exchange (replacing with 200 uL of fresh Endothelial Cell Growth Medium 2) was then performed by pipetor, and the plate was incubated for an additional 24 h. After this time (total duration ~64 h), most wells contained cells at confluence, and the assay proceeded as per the protocol with EA.hy926 cells. %Stimulation values were normalized to the response with 100 µM SLIGKV-NH_2 (100%).

f) Protocol for fibronectin binding assay

The fibronectin binding assay was performed as described previously.[3] HEK293FT cells were cultured in DMEM plus 10% FBS at 37 °C with 5% CO_2. Cells were co-transfected with αV and β3 integrin constructs with EndoFectin (GeneCopoeia) for at least 24 hours. Transfected cells were collected as described,[2] washed 3 times, and resuspended in HBSGB buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5.5 mM glucose, and 1% BSA)

25 µL of transfected HEK293FT cells were incubated with or without 5 or 50 µM of cRGD, MWM-319, or MWM-321 in HBSGB buffer containing 0.2 mM $CaCl_2$ and 2 mM $MnCl_2$ (Ca^{2+}/Mn^{2+}) at 25 °C for 10 min. Then, the Alexa Fluor 647-labeled Fn (15 µg/mL) and Alexa Fluor 488-labeled AP3 (anti-β3 mAb) were added to the cells. Ca^{2+}/Mn^{2+} condition was used to induce αVβ3 activation. As a control for the resting integrin state, cells were incubated with the Alexa Fluor 647-labeled Fn and Alexa Fluor 488-labeled AP3 in the HBSGB buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$

(Ca^{2+}/Mg^{2+}). The cells were incubated for another 30 min at 25 °C and analyzed by flow cytometry using a BD Accuri™ C6 flow cytometer. AP3-positive cells (expressing αVβ3 integrin) were acquired for calculating the mean fluorescence intensity (MFI) of AP3 binding (for integrin expression) and Fn binding. Fn binding was presented as normalized MFI, i.e. Fn MFI as a percentage of AP3 MFI (integrin expression).

g) Protocol for cell adhesion assay

48-well plates (MatTek Corporation, Ashland, MA) were coated overnight at 4 °C with 20 μg/mL human vitronectin (Vn, Sigma-Aldrich) in phosphate-buffered saline (PBS) at pH 7.4, followed by blocking with 1% BSA at 37 °C for 1 h.[4] HUVEC cells (1×10^6) were washed three times with DMEM and resuspended in 500 μL of DMEM. 100 μL aliquots of the HUVEC were treated with or without 50 μM cRGD, MWM-319, MWM-321, or DG-258 for 30 min. at 25 °C, and then seeded on the Vn-coated wells. After incubation at 37 °C for 1 h, cells were washed 3 times with PBS (pH 7.4) and fixed with 3.7% formaldehyde in PBS at 25° C for 5 min, and the nuclei were stained with 5 μg/mL DAPI. The stained cells were imaged with an EVOS digital inverted fluorescence microscope with a 10× objective.

4. Synthetic Protocols

a) General Information

All reagents and solvents were purchased from commercial vendors and used as received, unless noted. Reactions were performed in ventilated fume hoods with magnetic stirring and heated in oil baths, unless otherwise noted, and reactions were performed at room temperature (~20 °C) unless otherwise noted. Chilled reactions (below –10 °C) were performed in an acetone bath in a vacuum dewar, using a Neslab CC-100 immersion cooler. Deionized water was purified by charcoal filtration and used for reaction workups and in reactions with water. Unless otherwise noted, solutions were concentrated under reduced pressure using a rotary evaporator with Heidolph Rotovac vacuum pump, and final products, if non-volatile, were dried under high vacuum (typically <1 torr) using a Welch Duoseal 1400 belt-drive vacuum pump. Flash chromatography was performed using Biotage SNAP cartridges filled with 40–60 μm silica gel, or C18 reverse phase columns (Biotage® SNAP Ultra C18 or Isco Redisep® Gold C18Aq) on Biotage Isolera systems, with photodiode array UV detectors. Analytical thin layer chromatography (TLC) was performed on Agela Technologies 0.25 mm glass plates with 0.25 mm silica gel. Visualization was accomplished with UV light (254 nm) and aqueous potassium permanganate ($KMnO_4$) stain followed by heating, unless otherwise noted. NMR spectra were recorded on Varian 300 MHz or 400 MHz spectrometers as indicated. Proton and carbon chemical shifts are reported in parts per million (ppm; δ) relative to tetramethylsilane, $CDCl_3$ solvent, CD_3OD (^1H δ 0, ^{13}C δ 77.16, or ^{13}C δ 49.00, respectively). NMR data are reported as follows: chemical shifts, multiplicity (obs = obscured, app = apparent, br = broad, s = singlet, d = doublet, t = triplet, q = quartet, sxt = sextet, m = multiplet, comp = complex overlapping signals); coupling constant(s) in Hz; integration. Unless otherwise indicated, NMR data were collected at 25 °C. Tandem liquid chromatography/mass spectrometry (LC-MS) was performed on a Shimadzu LCMS-2020 with autosampler, photodiode array detector, and single-quadrupole MS with ESI and APCI dual ionization, using a Peak Scientific nitrogen generator. Unless otherwise noted, a standard LC-MS method was used to analyze reactions and reaction products: Phenomenex Gemini C18 column (100 x 4.6 mm, 3 μm particle size, 110 A pore size); column temperature 40 °C; 5 μL of sample in MeOH or CH_3CN at a nominal concentration of 1 mg/mL was injected, and peaks were eluted with a gradient of 25−95% CH_3CN/H_2O (both with 0.1% formic acid) over 5 min., then 95% CH_3CN/H_2O for 2 min. Purity was measured by UV absorbance at 210 or 254 nm. GC-MS was performed with a Shimadzu 2010 Plus GC with an AOC-20i auto injector and QP2010 SE MS detetector, and Shimadzu SH-5Rxi-4SiMS column (30 m, 0.25 mm ID, 0.25 μm film thickness). High-resolution mass spectra were obtained at the University of Cincinnati Environmental Analysis Service Center with an Agilent 6540 LCMS with accurate mass Q-TOF. IR spectra were obtained as a thin film on NaCl or KBr plates using a Thermo Scientific Nicolet iS5 spectrometer. Chemical names were generated and select chemical properties were calculated using either ChemAxon Marvin suite (https://www.chemaxon.com) or ChemDraw Professional 15.1. NMR data were processed using either MestreNova or ACD/NMR Processor Academic Edition (http://www.acdlabs.com) using the JOC report format.

b) Special materials

(3*S*)-4-(4-benzyloxyphenyl)-3-(*tert*-butoxycarbonylamino)butanoic acid (CAS# 126825-16-9, aka Boc-O-benzyl-L-β-homotyrosine) (**6**) was obtained from ChemImpex. (2*S*)-2-(*tert*-butoxycarbonylamino)-2-cyclohexyl-acetic acid (CAS# 109183-71-3, aka Boc-L-cyclohexylglycine) was obtained from Ark Pharm. (2*S*)-2-(*tert*-butoxycarbonylamino)-3-cyclohexyl-propanoic acid (CAS# 37736-82-6, aka Boc-b-cyclohexyl-L-alanine) (**21**) was obtained from ChemImpex. PEG spacers were obtained from MilliporeSigma or BroadPharm.

c) LC-MS Characterization Methods

Tandem liquid chromatography/mass spectrometry (LC-MS) was performed on a Shimadzu LCMS-2020 with autosampler, photodiode array detector, and single-quadrupole MS with ESI and APCI dual ionization using a Peak Scientific nitrogen generator.

Method A

Column: Phenomenex Gemini C_{18} (100 x 4.6 mm, 3 μm particle size, 110 Å pore size)
Column temperature: 40 °C
Sample Injection: 1-5 μL in MeCN or MeOH
Chromatographic monitoring: UV absorbance at 210 or 254 nm
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeOH w/ 0.1% formic acid
Flow Rate: 1.0 mL/min
Method: 0 to 0.1 min: 25% MeOH

0.1 min to 5 min: 25% to 95% MeOH
5 min to 7 min: 95% MeOH
7 min to 7.1 min: 95% to 25% MeOH
7.1 min to 9 min: 25% MeOH (equilibration)

Method B
Column: Phenomenex Gemini C_{18} (100 x 4.6 mm, 3 µm particle size, 110 Å pore size)
Column temperature: 40 °C
Sample Injection: 1-5 µL in MeCN or MeOH
Chromatographic monitoring: UV absorbance at 210 or 254 nm
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeOH w/ 0.1% formic acid
Flow Rate: 1.0 mL/min
Method: 0 to 0.5 min: 25% MeOH
0.5 min to 4 min: 25% to 95% MeOH
4 min to 6.3 min: 95% MeOH
6.3 min to 6.5 min: 95% to 25% MeOH
7.1 min to 9 min: 25% MeOH (equilibration)

Method C
Column: Phenomenex Gemini C_{18} (100 x 4.6 mm, 3 µm particle size, 110 Å pore size)
Column temperature: 40 °C
Sample Injection: 1-5 µL in MeCN or MeOH
Chromatographic monitoring: UV absorbance at 210 or 254 nm
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeCN w/ 0.1% formic acid
Flow Rate: 1.0 mL/min
Method: 0 to 0.1 min: 25% MeCN
0.1 min to 5 min: 25% to 95% MeCN
5 min to 7 min: 95% MeCN
7 min to 7.1 min: 95% to 25% MeCN
7.1 min to 9 min: 25% MeCN (equilibration)

Method D
Column: Phenomenex Gemini C_{18} (100 x 4.6 mm, 3 µm particle size, 110 Å pore size)
Column temperature: 40 °C
Sample Injection: 1-5 µL in MeCN or MeOH
Chromatographic monitoring: UV absorbance at 210 or 254 nm
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeOH w/ 0.1% formic acid
Flow Rate: 1.0 mL/min
Method: 0 to 0.5 min: 50% MeOH
0.5 min to 4 min: 50% to 95% MeOH
4 min to 9.5 min: 95% MeOH

d) Preparative HPLC Methods

Preparative liquid chromatography was performed on a Shimadzu LC-20AP preparative HPLC with autosampler, dual wavelength detector, and fraction collector.

Method A
Column: Phenomenex Gemini C_{18} Semi Preparative Column (250 x 10 mm, 5 µm particle size, 110 Å pore size)
Peak collection: measured by UV absorbance at 210 or 254 nm
Sample Injection: 0.1–1.9 mL (2 mL sample loop)
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeOH w/ 0.1% formic acid
Flow Rate: 6.0 mL/min
Method: 0 to 2.5 min: 40% MeOH
2.5 min to 6 min: 40% to 95% MeOH

6 min to 14 min: 95% MeOH
14 to 15 min: 95% to 40% MeOH
15 min to 16 min: 40% MeOH (equilibration)

Method B
Column: Phenomenex Gemini C_{18} Semi Preparative Column (250 x 10 mm, 5 µm particle size, 110 Å pore size)
Peak collection: measured by UV absorbance at 210 or 254 nm
Sample Injection: 0.1–1.9 mL (2 mL sample loop)
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeCN w/ 0.1% formic acid
Flow Rate: 6.0 mL/min
Method: 0 to 1.5 min: 25% MeCN
1.5 min to 7.5 min: 25% to 95% MeCN
7.5 min to 10.5 min: 95% MeCN

Method C
Column: Phenomenex Gemini C_{18} Semi Preparative Column (250 x 10 mm, 5 µm particle size, 110 Å pore size)
Peak collection: measured by UV absorbance at 210 or 254 nm
Sample Injection: 0.1–1.9 mL (2 mL sample loop)
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeOH w/ 0.1% formic acid
Flow Rate: 6.0 mL/min
Method: 0 to 3 min: 50% MeOH
3 min to 6.5 min: 25% to 95% MeOH
6.5 min to 12 min: 95% MeOH

Method D
Column: Phenomenex Gemini C_{18} Semi Preparative Column (250 x 10 mm, 5 µm particle size, 110 Å pore size)
Peak collection: measured by UV absorbance at 210 or 254 nm
Sample Injection: 0.1–1.9 mL (2 mL sample loop)
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeCN w/ 0.1% formic acid
Flow Rate: 6.0 mL/min
Method: 0 to 1.5 min: 25% MeCN
1.5 min to 6.5 min: 25% to 95% MeCN
6.5 min to 10.5 min: 95% MeCN

Method E
Column: Phenomenex Gemini C_{18} Semi Preparative Column (250 x 10 mm, 5 µm particle size, 110 Å pore size)
Peak collection: measured by UV absorbance at 210 or 254 nm
Sample Injection: 0.1–1.9 mL (2 mL sample loop)
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeOH w/ 0.1% formic acid
Method: 0 to 3 min: 50% MeOH
3 min to 4.5 min: 50% to 95% MeOH
4.5 min to 14 min: 95% MeOH

Method F
Column: Phenomenex Gemini C_{18} Semi Preparative Column (250 x 10 mm, 5 µm particle size, 110 Å pore size)
Peak collection: measured by UV absorbance at 210 or 254 nm
Sample Injection: 0.1–1.9 mL (2 mL sample loop)
Mobile Phase: Solvent A-H_2O w/ 0.1% formic acid, Solvent B-MeCN w/ 0.1% formic acid
Method: 0 to 0.75 min: 25% MeCN
0.75 min to 11.7 min: 25% to 95% MeCN
11.7 min to 12 min: 95% MeCN

e) General procedures for the solid phase peptide synthesis of compounds 4, 25b, 26b, and 27b

A CEM Liberty 1 synthesizer was used with a Discover microwave unit (North Carolina USA). Biosynthesis grade reagents were used, and amino acids were obtained from Anaspec or Novabiochem: Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-d-Phe-OH, Fmoc-Asn(Dmcp)-OH, Fmoc-propargylglycine, Fmoc-Orn(Boc)-OH.

Unless otherwise noted, the following reagents and solvents were used: DMF main solvent; amino acids were used as 0.2M solutions in NMP; Activator: 0.5M HATU in NMP; Activator base: 2.0M DIEA in NMP; Deprotection cocktail: 5% Piperazine in 9:1 NMP:EtOH. Resin: either 2-chlorotrityl chloride resin or preloaded Fmoc-Ser(tBu)-NovaSyn TGT, Fmoc-Thr(tBu)-NovaSyn TGT or Fmoc-Gly-NovaSyn TGT resin.

Microwave cycles were modified based on recommendations from CEM, using 5 eq. of amino acid, 5 eq. of activator, and 10 eq. of base. A coupling cycle of 50 °C max temp. for 10 min. was used for all amino acids, unless otherwise noted. In some cases, multiple cycles were required for complete reaction. Alternatively, we recommend considering the alternative use of COMU as coupling agent reported by Yamada and Shimizu for RGD peptides.[5] The resin was swollen in DCM for 15 min. before putting on the instrument, and the resin was manually washed on the instrument with DMF before synthesis. After synthesis, the resin was transferred to a tared all plastic syringe with PTFE frit, washed with DCM (20 mL), and dried overnight on the lyophilizer. A test cleavage was performed on a small amount of resin to verify the correct sequence before the complete cleavage was done for protected peptides. All couplings were done on instrument except for the Orn(Boc) coupling to the 2-chlorotrityl chloride resin which was done manually in the fume hood:

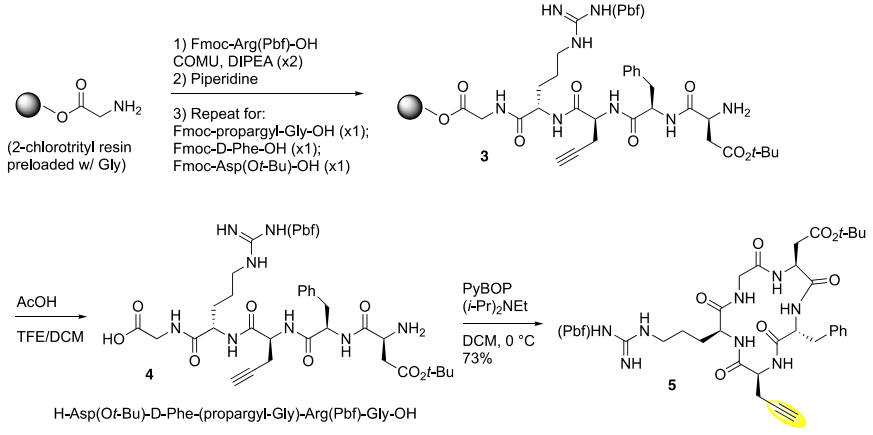
Orn(Boc) coupling based on modified protocol from JS Nowick lab, UC-Irvine: 300 mg resin/0.1 mmol scale; using 100 mg amino acid in DCM (8 mL) with 2,4,6-trimethylpyridine (0.3 mL). Briefly, the resin was swollen in DCM for 1 hour, the DCM was blown off, and the amino acid solution (0.2 M) and the 2,4,6-trimethylpyridine in DCM were added to the damp resin. The amino acid was coupled overnight, rocking at room. temp.; the resin was then washed in DCM before putting it on the instrument and performing subsequent cycles.

After synthesis, the resin was washed in DCM and dried. A test cleavage was performed with the cleavage cocktail [92.5% TFA, 2.5% triisopropylsilane, 2.5 % 3,6-dioxa-1,8-octanedithiol (DODT), 2.5% H_2O v/v] to verify the presence of the desired product by RP-HPLC and ESI-MS. HPLC was performed with a 50 x 4.6 mm Phenomenex Proteo C12 column, 5–90% MeCN/water gradient (with 0.1% TFA), 1 mL/min flow rate, 100 µL injected,] and LTQ ESI MS [direct injection, positive ion mode, ~2 µg of product injected] to verify test cleavage.

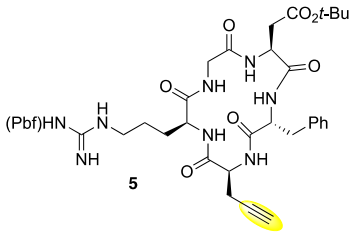
Protected peptides were cleaved from the resin with TFE:DCM (2:8) gassed with N_2, precipitated into cold ether, and centrifuged and washed 3x with cold ether, then the pellet was resuspended in 50% MeCN/water, frozen, and lyophilized. Peptides were purified by RP-HPLC [Vydac C8 228TP104 column, 20–90% or 40–90% MeCN/water gradient (with 0.1% TFA), 1 mL/min flow rate, 100 µL injected,], with Velos Orbitrap MS [200 µL sample in 70% MeCN/water with 0.1% formic acid, infusion, 5 µL/min; positive ion mode] to verify full length. Purified peptides were lyophilized.

f) Synthetic Protocols

Scheme 1. Synthesis of alkynyl-cilengitide analog 5



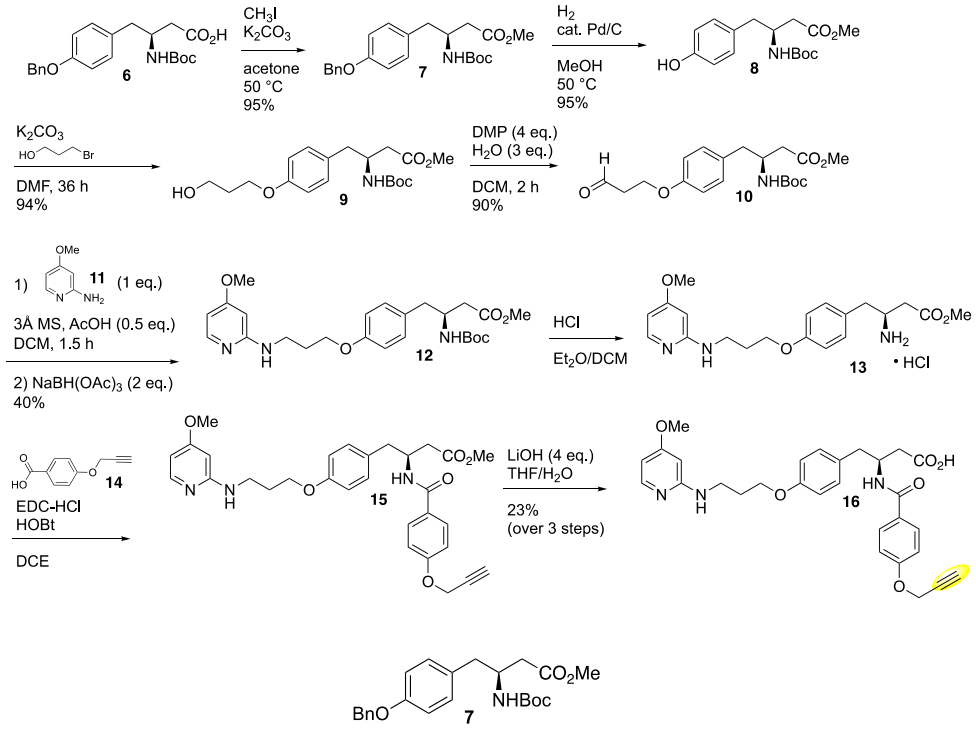
A portion of the crude linear peptide **4** (15 mg) was purified with preparative HPLC (Method E) to give 10.0 mg of pure material that was used in the subsequent cyclization reaction.



Cyclic peptide 5

To an oven dried 25 mL round bottomed flask with stir bar and under N_2 was added PyBOP (20.0 mg, 38.4 µmol), anhydrous DCM (12 mL), and DIPEA (5 µL, 29.2 µmol). The reaction was cooled in an ice bath and stirred for 15 min. under N_2. In a separate 5 mL vial was added the purified acyclic peptide **4** (10 mg, 11.1 µmol) and anhydrous DCM (2 mL). The mixture was sonicated for 1 min. to give a slightly turbid solution, and the solution was taken up into a 2 mL plastic syringe and added to the round bottomed flask dropwise using a syringe pump (0.1 mL/min. over 20 min). The reaction was stirred for 90 min. on ice and under N_2. An aliquot from the reaction was concentrated to give a colorless residue that was dissolved in HPLC grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated to give a yellow oil that was dissolved in DMSO (1 mL), syringe filtered (0.2 µm), and purified with preparative HPLC (Method C). Product fractions were pooled, concentrated under reduced pressure (to remove MeOH), and then lyophilized overnight to give **5** as a white solid (7.1 mg, 73%). LC/MS (Characterization Method C); m/z = 880.35 ($M + H^+$), m/z = 878.30 ($M - H^+$).

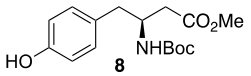
Scheme 2. Synthesis of alkynyl-modified Kessler ligand 15



Methyl (*S*)-4-(4-(benzyloxy)phenyl)-3-((*tert*-butoxycarbonyl)amino)butanoate (7)[6]

To a 250 mL RBF with stir bar, commercially available (3*S*)-4-(4-benzyloxyphenyl)-3-(*tert*-butoxycarbonylamino)butanoic acid (**6**) (0.800 g, 2.08 mmol) was added, followed by addition of freshly ground potassium carbonate (0.320 g, 2.32 mmol) and acetone (50 mL). The flask was sealed with a septum, and the reaction was flushed with nitrogen and stirred for 15 min., followed by addition of CH_3I (160 µL, 2.57 mmol), and the resultant

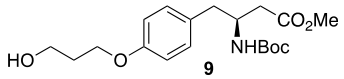
mixture was stirred for 3 h. A reflux condenser was then added and the reaction was heated to 50 °C for 16 h. A 0.1 mL sample was taken from the reaction, diluted with DCM in a microtube, and washed with 1N aq. HCl. The organic layer was separated, concentrated, and diluted with LC-MS grade methanol. TLC and LC-MS indicated complete consumption of starting material. The reaction was cooled to 20 °C and the solvent was evaporated. The resultant mixture was diluted with EtOAc (50 mL) and washed with half-saturated aq. sodium bicarbonate solution (50 mL). The organic layer was dried over sodium sulfate, filtered, and concentrated to yield ester **7** as a pale white waxy solid in high purity (822 mg, >95%). TLC: 30% EtOAc/hexanes, R_f 0.62; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.24 (comp, 5H), 7.10 (d, *J* = 8.1 Hz, 2H), 6.94 – 6.87 (m, 2H), 5.04 (s, 3H), 4.12 (br s, 1H), 3.68 (s, 3H), 2.96 – 2.64 (m, 2H), 2.47 (qd, *J* = 15.9, 5.6 Hz, 2H), 1.41 (s, 9H); ^{13}C NMR (101 MHz , $CDCl_3$) δ 157.6, 155.1, 137.0, 130.4, 130.0, 128.6, 127.9, 127.5, 114.8, 79.3, 70.0, 51.7, 48.9, 39.5, 37.4, 28.4; LC/MS t_R = 6.64 min (Characterization Method A); MS (ESI) for $C_{23}H_{29}NO_5$: calculated m/z = 421.96 ($M+H^+$), 299.46 (M–Boc); observed m/z = 421.90 ($M+H^+$), 299.85 (M–Boc).



8

Methyl (*S*)-3-((*tert*-butoxycarbonyl)amino)-4-(4-hydroxyphenyl)butanoate (8)[6]

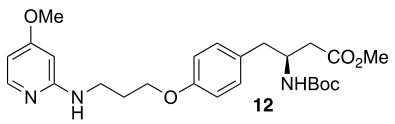
To 250 mL hydrogenator flask with stir bar, benzyl ether **7** (800 mg, 2.00 mmol) was added, followed by MeOH (50 mL). The flask was covered with parafilm and flushed with nitrogen for 2 min. To 8 ml vial, A suspension of Pd/C (10%, 220 mg, 0.21 mmol) in water (3 mL) was prepared in a 8 mL vial, and transferred to the solution of **7,** and the flask was immediately connected to a Parr hydrogenator. The reaction was evacuated and flushed with hydrogen 3x, and the resultant black suspension was stirred under H_2 at 44 psi for 2 h at 20 °C. After 2 h, a sample was taken from the reaction, passed through the plug of Celite and cotton, concentrated, and then diluted with LC-MS grade methanol. TLC and LC-MS analysis of sample showed complete consumption of starting material; therefore the rest of reaction was passed through a plug of Celite and cotton, and washed with MeOH (3 x 50 mL). The filtrate was concentrated to yield a crude oil (0.62 g). The oil was dissolved in DCM and purified by flash chromatography (50 g SiO_2 cartridge; 0–100% EtOAc/hexanes gradient) to yield the phenol **8** as a pale yellow oil (593 mg, >95%). TLC: 40% EtOAc/hexanes, R_f 0.52; ^{1}H NMR (300 MHz ,$CDCl_3$) δ 7.00 (dd, J = 8.6, 2.3 Hz, 2H), 6.77 – 6.71 (m, 2H), 6.32 (s, 1H), 5.13 (d, *J* = 9.3 Hz, 1H), 3.68 (d, *J* = 2.5 Hz, 3H), 2.88 – 2.65 (m, 2H), 2.46 (qd, *J* = 15.9, 5.7 Hz, 2H), 1.46 – 1.33 (s, 9H); ^{13}C NMR (101 MHz , $CDCl_3$) δ 172.6, 155.8, 155.5, 130.5, 128.8, 115.7, 60.8, 52.0, 49.3, 39.8, 28.5; LC/MS t_R = 5.25 min. (Characterization Method A); MS (ESI) for $C_{16}H_{23}NO_5$: calculated m/z = 310.36 ($M+H^+$), 209.36 (M-Boc), 641.72 ($2M+Na^+$); observed m/z = 309.90 ($M+H^+$), 209.80 (M-Boc), 641.15 ($2M+Na^+$).



9

Methyl (*S*)-3-((*tert*-butoxycarbonyl)amino)-4-(4-(3-hydroxypropoxy)phenyl)butanoate (9)[6]

To a 250 mL RBF with stir bar, phenol **8** (780 mg, 2.52 mmol), freshly powdered K_2CO_3 (875 mg, 6.33 mmol), and dry DMF (50 mL) were added. The flask was sealed with a septum and flushed briefly with nitrogen. The resultant mixture was stirred 30 min., followed by addition of 3-bromopropan-1-ol (300 µL, 3.32 mmol), and the resultant suspension was stirred for 15 h. A 0.1 mL sample was taken from the reaction and diluted in a microtube with DCM and 1 M HCl. The organic layer was concentrated and diluted with LC-MS grade methanol. TLC and LC-MS analysis indicated complete consumption of starting material. The reaction was diluted with 1M aq. HCl (150 mL) and extracted with EtOAc (3 x 150 mL), and the combined organics were washed with brine (100 mL), dried over sodium sulfate, filtered, and concentrated to yield a crude oil (1.02 g). The oil was dissolved in DCM and purified by flash chromatography (25 g SiO_2 cartridge; 0–15% MeOH/DCM gradient) to yield 872 mg (94%) alcohol **3** as a pale yellow oil with some impurities visible by proton NMR. TLC: 10% MeOH/DCM, R_f 0.5; LC/MS t_R = 5.25 min. (Characterization Method A); MS (ESI) for $C_{19}H_{29}NO_6$: calculated m/z = 389.94 ($M+Na^+$), 267.44 (M-Boc), 757.38 ($2M+Na^+$); observed m/z = 389.90 ($M+Na^+$), 267.90 (M-Boc), 757.26 ($2M+Na^+$). Alcohol **9** was pushed to the next step without additional purification.

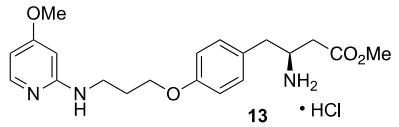


12

Methyl (*S*)-3-((*tert*-butoxycarbonyl)amino)-4-(4-(3-((4-methoxypyridin-2-yl)amino)propoxy)phenyl)butanoate (12).[6]

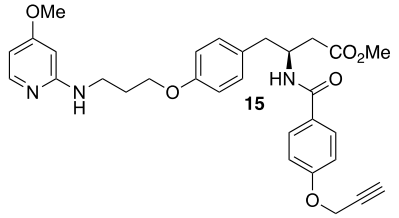
Part I: Oxidation: To a 100 mL RBF with stir bar, alcohol **9** (800 mg, 2.18 mmol), DCM (40 mL) and DMP (2.00 g, 4.36 mmol) were added. The resultant yellow colored suspension was stirred for 1 h, followed by addition of water (118 µL, 6.53 mmol). The reaction was stirred for another 1.5 h, after which time a sample was taken from the reaction, quenched with 10% aq. sodium thiosulfate solution in a microtube, and diluted with DCM. The organic phase was concentrated and diluted with LC-MS grade methanol, and LC-MS analysis showed completion of the reaction. The reaction was then quenched with 10% aq. sodium thiosulfate (50 mL), separated, and the aqueous layer was re-extracted with DCM (2 x 20 mL). The combined organics were washed with saturated sodium bicarbonate solution (50 mL) and brine (50 mL), then dried over sodium sulfate, filtered, and concentrated to yield aldehyde **10** (1.00 g) as a yellow oil.

Part II: Reductive amination: To a 250 mL RBF with stir bar, aldehyde **10** (0.790 mg), 3Å molecular sieves, 2-amino-4-methoxypyridine (**1**) (322 mg, 2.59 mmol), dry DCM (45 mL), and conc. HCl (0.45 mL) were added (N.B. HCl was added mistakenly instead of acetic acid, nonetheless the reaction proceeded in acceptable yield). The flask was sealed under N_2, and the resultant mixture was stirred for 1.5 h. Sodium triacetoxyborohydride (460 mg, 2.16 mmol) was added, and the reaction was stirred for 3.5 h. Saturated sodium bicarbonate solution (10 mL) was added slowly to neutralize the reaction, which was then diluted with water (30 mL). The organic phase was washed with brine (20 mL), dried over sodium sulfate, filtered, and concentrated to yield a pale yellow oil (760 mg). The crude was dissolved in DMSO and loaded to a 30 g C18 column and eluted with a 15–100 % MeOH/water (both with 0.1% formic acid) gradient to give aminopyridine **4** as a colorless oil (425 mg, 40% yield over 2 steps). ^{1}H NMR (400MHz, $CDCl_3$) δ 9.64 (br s, 1H), 8.57 (br s, 1H), 7.55 (d, *J* = 6.8 Hz, 1H), 7.06 (d, *J* = 8.2 Hz, 2H), 6.79 (d, *J* = 8.1 Hz, 2H), 6.18 (dd, *J* = 6.9, 2.1 Hz, 1H), 5.94 (d, *J* = 2.1 Hz, 1H), 5.04 (d, *J* = 9.0 Hz, 1H), 4.04 (t, *J* = 5.5 Hz, 2H), 3.77 (s, 3H), 3.66 (s, 3H), 3.40 (t, *J* = 6.7 Hz, 2H), 2.88 – 2.66 (m, 2H), 2.43 (qd, *J* = 15.8, 5.6 Hz, 2H), 2.19 – 2.10 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.9, 169.9, 157.2, 155.0, 139.5, 130.3, 130.1, 114.3, 102.4, 88.8, 79.2, 64.5, 55.7, 51.6, 48.9, 39.5, 39.0, 37.4, 28.4, 28.2; LC/MS t_R = 5.44 min (Characterization Method A); MS (ESI) for $C_{25}H_{35}N_3O_6$: calculated m/z = 474.25 ($M+H^+$), observed m/z = 474.40 ($M+H^+$).



Methyl (S)-3-amino-4-(4-(3-((4-methoxypyridin-2-yl)amino)propoxy)phenyl)butanoate hydrochloride (13)[6]

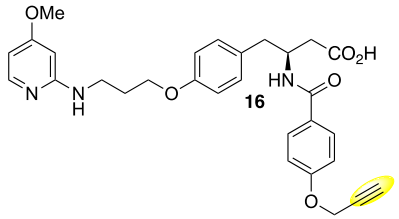
Carbamate **12** (36.0 mg, 0.081 mmol) was added to a 20 mL vial with stir bar, and the vial was sealed with a septum and flushed with nitrogen. Dry DCM (2 mL) and 4.0 M HCl in dioxane (2 mL) were added by syringe, and the reaction was stirred for 16 h. The reaction was concentrated via a rotary evaporator with a connected base trap, to yield the amine HCl salt **5**, which was used in the next step without purification. 1H NMR showed residual dioxane at 3.66 ppm; 2 peaks were observed in LC-MS at 1.00 min and 1.33 min due to solubility issues of **5**. ^{1}H NMR (400 MHz, CD_3OD) δ 7.70 (d, *J* = 7.2 Hz, 1H), 7.19 (d, *J* = 7.9 Hz, 1H), 6.94 (d, *J* = 8.0 Hz, 1H), 6.54 – 6.46 (m, 1H), 6.42 (d, *J* = 2.3 Hz, 1H), 4.13 (t, *J* = 5.7 Hz, 2H), 3.94 (s, 3H), 3.80 – 3.66 (comp, 5H), 3.62 – 3.54 (comp, 3H), 3.01 (dd, *J* = 13.9, 6.0 Hz, 1H), 2.86 (dd, *J* = 13.9, 8.4 Hz, 1H), 2.65 (dd, *J* = 11.6, 6.0 Hz, 2H), 2.22 – 2.10 (m, 2H); LC/MS t_R = 1.00 min (Characterization Method A); MS (ESI) for $C_{19}H_{25}N_3O_4$: calculated m/z = 374.25 ($M+H^+$), observed m/z = 373.95 ($M+H^+$).



Methyl (*S*)-4-(4-(3-((4-methoxypyridin-2-yl)amino)propoxy)phenyl)-3-(4-(prop-2-yn-1-yloxy)benzamido)butanoate (15)

To 4 mL vial with stir bar containing amine **13** (30 mg, 0.073 mmol) was added 4-propargyloxybenzoic acid (**14**) (12.9 mg, 0.073 mmol, synthesized using the published protocol[7]), EDC HCl (16.8 mg, 0.088 mmol), and HOBt (11.9 mg, 0.088 mmol). The vial was sealed and flushed under N_2. NEt_3 (25 µL, 0.18 mmol) and dry DCM (4 mL) were added and the reaction was stirred for 16 h. The reaction was then diluted with EtOAc (10 mL), washed with half-saturated sodium bicarbonate (15 mL), dried over sodium sulfate, filtered, and concentrated. The resulting crude was dissolved with HPLC grade DMSO, filtered through a PTFE 0.22 µM syringe filter, and purified with preparative HPLC (Method A). Product fractions were pooled, concentrated under reduced pressure (to remove MeOH), and lyophilized overnight to give amide

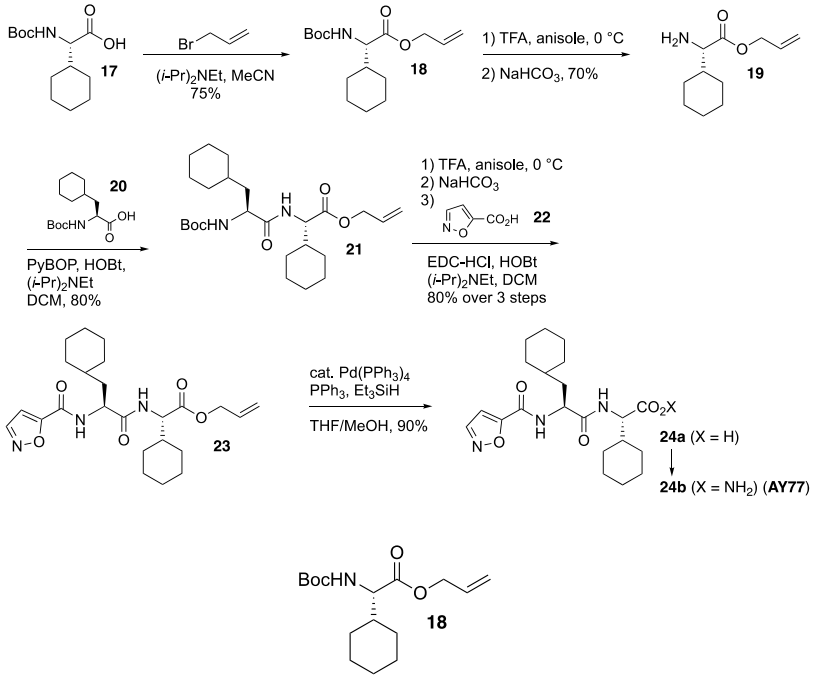
15 as a colorless oil (20 mg, 52% yield over 2 steps). ^{1}H NMR (400 MHz, CD_3OD) δ 8.48 (s, 1H), 7.67 (dd, *J* = 15.6, 7.7 Hz, 3H), 7.15 (d, *J* = 8.3 Hz, 2H), 7.00 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* = 8.3 Hz, 2H), 6.32 (dd, *J* = 6.8, 2.2 Hz, 1H), 6.18 (d, *J* = 2.3 Hz, 1H), 4.77 (d, *J* = 2.4 Hz, 2H), 4.59 (p, *J* = 7.0 Hz, 1H), 4.04 (t, *J* = 5.9 Hz, 2H), 3.82 (s, 3H), 3.62 (s, 3H), 3.47 (t, *J* = 6.7 Hz, 2H), 2.98 (t, *J* = 2.4 Hz, 1H), 2.84 (d, *J* = 7.2 Hz, 2H), 2.60 (d, *J* = 6.8 Hz, 2H), 2.07 (p, *J* = 6.5 Hz, 2H); ^{13}C NMR (101 MHz, CD_3OD) δ 172.1, 169.2, 168.2, 167.8, 160.3, 157.6, 157.5, 140.9, 130.3, 130.0, 128.6, 127.2, 114.2, 114.1, 103.3, 90.8, 77.9, 75.8, 64.8, 55.3, 55.1, 50.8, 48.8, 39.1, 39.0, 38.7, 38.1, 28.4; LC/MS t_R = 3.76 min (Characterization Method A); MS (ESI) for $C_{30}H_{33}N_3O_6$: calculated m/z = 532.61 ($M+H^+$), observed m/z = 532.05 ($M+H^+$).



16

(*S*)-4-(4-(3-((4-methoxypyridin-2-yl)amino)propoxy)phenyl)-3-(4-(prop-2-yn-1-yloxy)benzamido)butanoic acid (16)
To 4 mL vial with stir bar containing ester **15** (20 mg, 0.038 mmol) was added LiOH-H_2O (8.00 mg, 0.191 mmol). The vial was sealed and flushed with N_2. THF (1.5 mL) and water (0.5 mL) were added. The mixture was vigorously stirred for 18 h. The reaction was quenched with 0.1 M HCl (1 mL), and the volume was reduced with rotary evaporator. The crude was dissolved in HPLC grade DMSO, filtered through a PTFE 0.22 μm syringe filter, and purified with preparative HPLC (Method A). Product fractions were pooled, concentrated under reduced pressure (to remove MeOH), and lyophilized overnight to give **7** as a white solid (11 mg, 52%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.68 (comp, 3H), 7.15 (d, *J* = 7.9 Hz, 2 H), 6.99 (d, *J* = 7.6 Hz, 2 H), 6.80 (d, *J* = 7.7 Hz, 2 H), 6.26 (d, *J* = 6.4 Hz, 1 H), 6.11 (s, 1 H), 4.76 (s, 2 H), 4.02 (t, *J* = 5.7 Hz, 2 H), 3.78 (s, 3 H), 3.50 (d, *J* = 5.9 Hz, 5 H), 2.95 (s, 1 H), 2.85 (br s, 1 H), 2.64 (s, 1 H), 2.04 (t, *J* = 6.2 Hz, 2 H); ^{13}C NMR (101 MHz , DMSO-d_6) δ 173.3, 166.9, 166.1, 165.2, 160.4, 159.5, 157.2, 147.9, 131.0, 130.5, 129.4, 127.6, 114.8, 114.4, 101.8, 91.2, 79.3, 78.7, 65.6, 55.9, 55.3, 48.8, 29.0; LC/MS t_R = 2.83 min (Characterization Method A); MS (ESI) for $C_{29}H_{31}N_3O_6$: calculated m/z = 518.58 ($M+H^+$), observed m/z = 518.05 ($M+H^+$). HRMS (ESI-TOF) calculated for $C_{29}H_{31}N_3O_6$: 518.2286, found 518.2247.

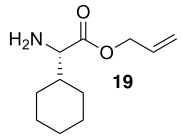
Scheme 3. Synthesis of the acid derivative of the PAR2 agonist AY77



Allyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-2-cyclohexylacetate (18)

To an oven dried round bottomed flask with stir bar and under nitrogen was added (2*S*)-2-(*tert*-butoxycarbonylamino)-2-cyclohexyl-acetic acid (**17**) (1.5 g, 5.8 mmol) and anhydrous MeCN (45 mL). Allyl bromide

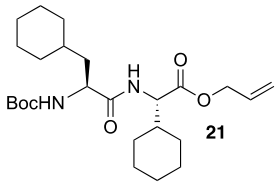
(0.60 mL, 6.9 mmol), and DIPEA (1.1 mL, 6.3 mmol) were added and the reaction was stirred for 20 h at room temperature. The reaction was monitored with TLC and the product was visible with $KMnO_4$ stain (R_f = 0.8 using 50% EtOAc/hexanes). The reaction was then concentrated under reduced pressure to give crude material that was dissolved in EtOAc (100 mL) and washed with H_2O (75 mL, x 2), saturated $NaHCO_3$ (x 2), brine, dried with $MgSO_4$, filtered, and concentrated under reduced pressure to give ester **18** as a colorless oil in 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.99 - 5.82 (m, 1H), 5.38 (ddd, *J* = 1.5, 12.6 Hz, 1H), 5.29 - 5.20 (m, 1H), 5.03 (br d, *J* = 8.8 Hz, 1H), 4.69 - 4.57 (m, 2H), 4.22 (dd, *J* = 4.4, 5.3 Hz, 1H), 1.83 – 0.97 (comp, 11H), 1.44 (s, 9H).



19

Allyl (*S*)-2-amino-2-cyclohexylacetate (19)

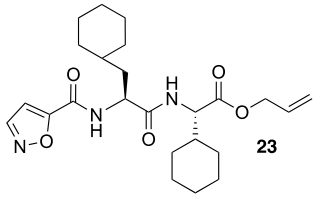
To a round bottomed flask with stir bar, under nitrogen, and containing ester **18** (1.3 g, 4.2 mmol) was added anhydrous DCM (40 mL) and anisole (1.3 mL, 13 mmol). The reaction was cooled in an ice bath for 10 min, then TFA (5 mL) was added dropwise over 1 min., and the reaction was stirred on ice for 3 h. The reaction was monitored with TLC using ninhydrin or $KMnO_4$ to visualize product. After completion, the reaction was concentrated under reduced pressure (with base trap connected to vacuum pump) to give a crude TFA salt. This was dissolved in EtOAc (50 mL) and washed with $NaHCO_3$ (x 2), brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude free amine was dry loaded using silica onto a 50 g SiO_2 column and purified with flash chromatography (100% EtOAc) to give amine **19** as a colorless oil (578 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.99 - 5.85 (m, 1H), 5.34 (d, *J* = 17.2 Hz, 1H), 5.26 (d, *J* = 10.2 Hz, 1H), 4.63 (d, *J* = 5.5 Hz, 2H), 3.31 (d, *J* = 4.7 Hz, 1H), 1.77 – 0.99 (comp, 11H); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 175.3, 132.0, 118.5, 65.3, 59.6, 42.1, 29.7.



21

Allyl (*S*)-2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-cyclohexylpropanamido)-2-cyclohexylacetate (21)

To an oven dried round bottomed flask with stir bar, under nitrogen, and containing amine **19** (550 mg, 2.80 mmol) was added (2*S*)-2-(*tert*-butoxycarbonylamino)-3-cyclohexyl-propanoic acid (**20**) (900 mg, 3.30 mmol) and anhydrous DCM (40 mL). PyBOP (1.6 g, 3.1 mmol), HOBt (640 mg, 4.2 mmol), and DIPEA (0.5 mL, 2.9 mmol) were added consecutively and the reaction was stirred for 21 h under nitrogen. The reaction was monitored with TLC, using $KMnO_4$ to visualize the product. After completion, the reaction was concentrated under reduced pressure, re-dissolved in EtOAc (120 mL), and washed with saturated aq. $NaHCO_3$ (x 2), brine, dried over $MgSO_4$, filtered, and concentrated to give crude material that was dry loaded using SiO_2 and purified with column chromatography (SiO_2; EtOAc/hexanes 10–50%) to give amide as a white solid **21** (1.04 g, 80%). mp = 119-120 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.54 (d, *J* = 8.2 Hz, 1H), 5.98 - 5.84 (m, 1H), 5.34 (dd, *J* = 1.2, 17.2 Hz, 1H), 5.26 (dd, *J* = 1.4, 10.4 Hz, 1H), 4.86 (d, *J* = 8.2 Hz, 1H), 4.63 (dd, *J* = 1.2, 5.9 Hz, 2H), 4.54 (dd, *J* = 5.3, 8.8 Hz, 1H), 4.18 - 4.09 (m, 1H), 1.88 - 1.55 (comp, 11H), 1.50 - 1.39 (m, 9H), 1.37 - 0.82 (comp, 13H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.2, 171.3, 131.5, 118.8, 65.7, 56.7, 52.3, 41.0, 39.3, 33.9, 33.5, 32.7, 29.4, 28.2, 27.9, 26.3, 26.1, 26.0, 25.93, 25.92, 25.89.



23

Allyl (*S*)-2-cyclohexyl-2-((*S*)-3-cyclohexyl-2-(isoxazole-5-carboxamido)propanamido)acetate (23)

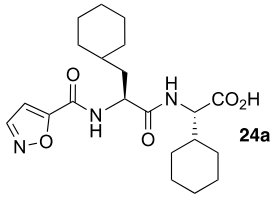
Part 1: Boc removal

Anhydrous DCM (15 mL) was added to a round bottomed flask with stir bar containing carbamate **21** (850 mg, 1.89 mmol), sealed under nitrogen. The solution was cooled in an ice bath for 10 min., then TFA (4.3 mL) was added dropwise over 1 min., and the reaction was stirred on ice. The reaction was monitored with TLC, using either $KMnO_4$ or iodine on

silica gel to visualize the product. After 3 h, the reaction was concentrated under reduced pressure to give the crude TFA salt. This was dissolved in EtOAc (100 mL) and washed with saturated $NaHCO_3$ (3 x 50 mL), brine, dried over $MgSO_4$, filtered, and concentrated to give the crude free amine (890 mg) as a yellow oil.

Part 2: Coupling

Approximately 700 mg of the crude oil was added to a round bottomed flask with stir bar and placed under nitrogen. The crude material was dissolved in anhydrous DCM (25 mL) and isoxazole-5-carboxylic acid (**20**) (248 mg, 2.19 mmol), EDC•HCl (574 mg, 2.99 mmol), HOBt (459 mg, 2.99 mmol), and DIPEA (0.38 mL, 2.2 mmol) were added, and the reaction was stirred for 17 h under nitrogen. A sample aliquot was taken from the reaction, concentrated under reduced pressure, dissolved in HPLC grade MeOH and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated and then re-dissolved in EtOAc (75 mL), washed with H_2O (2 x 50 mL), saturated aq. $NaHCO_3$ (x 2), brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give crude material that was dry loaded with SiO_2 onto a 50 g SiO_2 column and purified with flash chromatography (SiO_2; 10 to 60% EtOAc/hexanes) to give amide **23** as a white solid (668 mg, 80% yield over 2 steps). mp = 126-128° C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.35 (d, *J* = 2.0 Hz, 1H), 7.11 (d, *J* = 8.6 Hz, 1H), 6.95 (d, *J* = 1.6 Hz, 1H), 6.48 (d, *J* = 8.6 Hz, 1H), 5.97 - 5.86 (m, 1H), 5.35 (ddd, *J* = 1.4, 17.2 Hz, 1H), 5.28 (ddd, *J* = 1.3, 10.2 Hz, 1H), 4.75 - 4.67 (m, 1H), 4.67 - 4.62 (m, 2H), 4.56 (dd, *J* = 5.1, 8.6 Hz, 1H), 1.87 - 0.83 (comp, 22H); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 171.2, 170.8, 162.3, 155.5, 151.0, 131.4, 119.0, 106.7, 65.9, 56.9, 51.1, 40.9, 39.9, 33.9, 33.5, 32.7, 29.4, 28.0, 26.2, 26.0, 25.9, 25.87, 25.85.

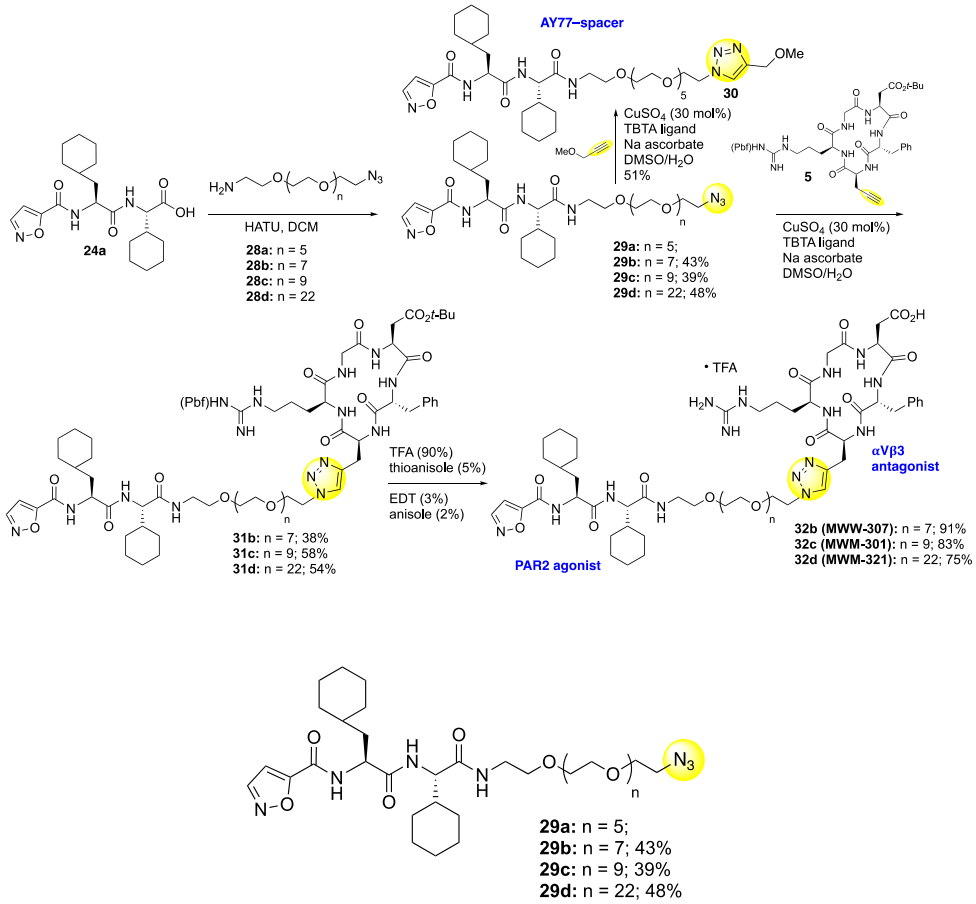


24a

(*S*)-2-Cyclohexyl-2-((*S*)-3-cyclohexyl-2-(isoxazole-5-carboxamido)propanamido)acetic acid (24a)

To an oven dried round bottomed flask with stir bar and under nitrogen was added allyl ester **23** (250 mg, 0.561 mmol), anhydrous THF (8 mL), and anhydrous MeOH (8 mL). Triphenylphosphine (25 mol%, 35 mg), Et_3SiH (0.18 mL, 0.11 mmol), and $Pd(PPh_3)_4$ (10 mol%, 65.0 mg, 0.056 mmol) were added, and the reaction was under nitrogen. A sample aliquot was taken from the reaction, concentrated, dissolved in HPLC grade MeOH, and analyzed with LC-MS to confirm reaction completion. After 3 h, the reaction was concentrated to give crude material that was dry loaded using SiO_2 onto a 25 g SiO_2 column and partially purified with flash chromatography (0 to 20% MeOH/DCM) to give **24a** as a white solid (206 mg, 90% yield). The product still contained a small trace of triphenylphosphine oxide. A small batch (12 mg) was triturated with hexanes/ether (60/40) to give highly pure product (7 mg). mp = 126-128 °C; LC/MS t_R = 6.25 min (Characterization Method B); m/z = 406.90 ($M + H^+$), m/z = 404.05 ($M - H^+$). ^{1}H NMR (300 MHz, CD_3OD) δ 8.52 (s, 1H), 7.01 (s, 1H), 4.72 (app t, *J* = 7.6 Hz, 1H), 4.31 (d, *J* = 5.9 Hz, 1H), 1.89 - 1.58 (comp, 14 H), 1.45 - 0.90 (comp, 10H); ^{13}C NMR (75 MHz, CD_3OD) δ 173.6, 173.0, 162.5, 156.8, 151.1, 106.2, 57.6, 51.5, 40.2, 38.9, 34.2, 33.5, 32.3, 29.6, 28.2, 26.3, 26.1, 26.06, 26.01, 25.9.

Scheme 4. Assembly of heterobivalent ligands featuring cilengitide analog and AY77



AY77-PEG azide 29b

To an oven dried 5 mL vial with stir bar and under nitrogen was added acid **24a** (10.0 mg, 24.7 µmol), 26-azido-3, 6, 9, 12, 15, 18, 21, 24-octaoxahexacosan-1-amine (**28b**) (12 mg, 34.2 µmol), and HATU (14.5 mg, 37.5 µmol). Anhydrous DCM (3 mL) was added and the reaction was stirred for 90 min. under nitrogen. An aliquot was taken from the reaction, concentrated, dissolved in HPLC MeCN, and analyzed with LC-MS to confirm reaction completion. LC-MS showed a mix of product and starting material. Additional HATU (7.2 mg, 18.7 µmol) was added and the reaction was stirred for an additional 1 hr, after which time LC-MS completion of the reaction. The reaction was concentrated to give a yellow oil that was dissolved in DMSO (~1 mL), filtered, and purified with preparative HPLC (Method C). Product fractions were pooled, concentrated under reduced pressure (to remove MeOH), and lyophilized overnight to give **29b** as a colorless oil (8.8 mg, 43%). LC/MS t_R = 6.40 min (Characterization Method A); m/z = 824.30 (M - H^+); 826.40 (M + H^+), 848.40 (M + Na^+).

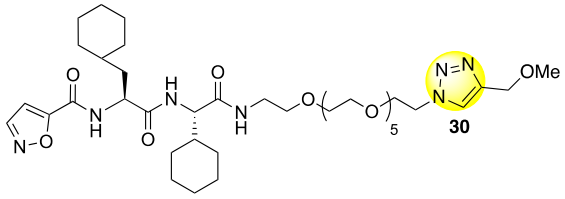
AY77-PEG azide 29c

To an oven dried 5 mL vial under nitrogen with stir bar was added acid **24a** (10 mg, 25 µmol), 32-azido-3, 6, 9, 12, 15, 18, 21, 24, 27, 30-decaoxadotriacontan-1-amine (**28c**) (19.5 mg, 37 µmol), and HATU (25 mg, 66 µmol). Anhydrous DCM (1 mL) was added and the reaction was stirred for 1 h. An aliquot of the reaction was concentrated to give an oil that was dissolved in HPLC grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated to give crude material that was dissolved in DMSO (1.5 mL), filtered, and purified with preparative HPLC (Method C). Pooled fractions were concentrated under reduced pressure (to remove MeOH) and then lyophilized overnight to give amide **29c** as a colorless oil (8.7 mg, 39%). LC/MS t_R = 5.11 (Characterization Method C); m/z = 914.50 (M + H^+), m/z = 912.50 (M – H^+).

AY77-PEG-azide 29d

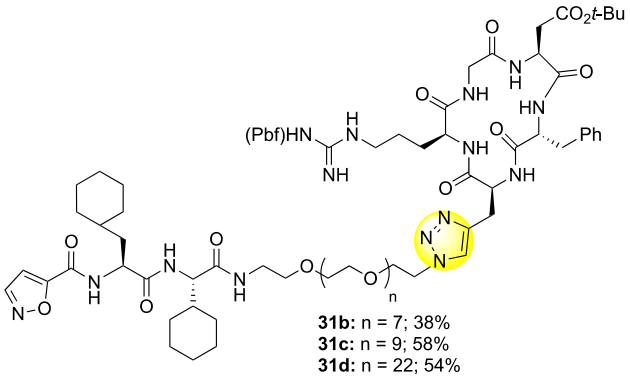
To an oven dried 20 mL vial with stir bar and under nitrogen was added acid **24a** (6 mg, 15 µmol), 71-azido-3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, 60, 63, 66, 69-tricosaoxahenheptacontan-1-amine (**28d**) (24.4 mg, 22.2 µmol), and HATU (20 mg, 52 µmol). Anhydrous DCM (1.5 mL) was added and the reaction was stirred for 4 h under nitrogen. An aliquot of the reaction was concentrated under reduced pressure to give a yellow residue that was dissolved

in HPLC grade MeCN and analyzed with LC-MS to verify reaction completion. The reaction was concentrated under reduced pressure in lukewarm water (i.e. no heating of rotovap bath) to give a yellow oil. The crude material was dissolved in DMSO (1.0 mL), filtered, and purified with preparative HPLC (Method B). Product fractions were pooled together, concentrated under reduced pressure (to remove MeOH), and then lyophilized overnight to give amide **29d** as a colorless oil (10.1 mg, 48%). LC/MS t_R = 4.86 (Characterization Method C); m/z = 1486.85 ($M + H^+$), m/z = 1484.80 ($M - H^+$).



AY77-PEG control ligand 30

To a 5 mL vial with stir bar was added **29a** (2.5 mg, 3.4 µmol) and propargyl methyl ether (0.340 mL of a 0.02 M solution in DMSO). In a separate 5 mL vial was added sodium ascorbate (2.0 mg, 10 µmol), TBTA (3.6 mg, 6.8 µmol), and $CuSO_4$ (30 mol%, 50 µL of a 0.02 M solution in H_2O). DMSO (0.4 mL) was added, the mixture was sonicated for 20 s, and nitrogen was bubbled through the resulting solution for 30 s. This solution was then added to the first vial, additional DMSO (0.5 mL) was added, nitrogen was bubbled through the reaction for 20 s, and the reaction was stirred at room temperature under nitrogen. After 2 h, 2 drops of the reaction were diluted with HPLC grade MeCN, and the reaction was analyzed with LC-MS to confirm reaction completion. The reaction was syringe filtered (0.2 µm) and purified with preparative HPLC (Method B). Product fractions were pooled together and concentrated at room temperature (to remove MeOH), and then lyophilized overnight to give **30** as a white film (1.4 mg, 51%). LC/MS t_R = 4.55 min (Characterization Method C); m/z = 808.50 ($M + H^+$), m/z = 806.40 ($M - H^+$); HRMS calcd. for $C_{39}H_{65}N_7O_{11}$ ($M + H^+$) 808.4815, found 808.4802.



Protected bivalent ligand AY77-PEG-cilengitide 31b

To a 20 mL with stir bar was added azide **29b** (6.5 mg, 7.9 µmol) and cilengitide-alkyne **5** (5.5 mg, 6.3 µmol). In a separate 5 mL vial was added sodium ascorbate (3.7 mg, 19 µmol), TBTA (4.2 mg, 7.9 µmol), and $CuSO_4$ (2 µmol, 0.1 mL of a 0.02 M solution in H_2O, 30 mol%). DMSO (0.5 mL) was added, the mixture was sonicated for 10 s, and nitrogen was bubbled through the resulting solution for 30 s. This solution was then added to the first vial, additional DMSO (1.0 mL) was added, nitrogen was bubbled through the reaction for 20 s, and the reaction was stirred under nitrogen. After 1 h, 1 drop of the reaction was diluted with HPLC grade MeCN and the reaction was analyzed with LC-MS to confirm reaction completion. The reaction was filtered and purified with preparative HPLC (Method D). Product fractions were pooled and concentrated at room temperature (to remove MeCN), and then lyophilized overnight to give **31b** as a colorless oil (4 mg, 38%). LC/MS t_R = 5.67 min (Characterization Method C); m/z = 1706.05 ($M + H^+$); 1704.00 ($M - H^+$); 1749.0 (formate adduct).

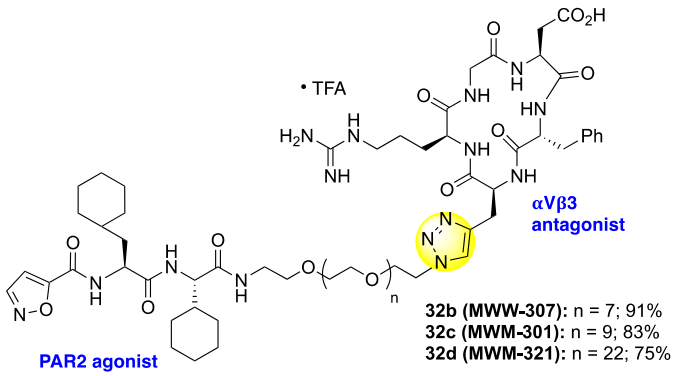
Protected bivalent ligand AY77-PEG-cilengitide 31c

To a 5 mL vial with stir bar was added azide **29c** (7.5 mg, 8.2 µmol) and cilengitide-alkyne **5** (5.9 mg, 6.7 µmol). In a separate 5 mL vial was added sodium ascorbate (4 mg, 20 µmol), TBTA (4.5 mg, 8.5 µmol), and $CuSO_4$ (1.08 µmol, 0.1

mL of a 0.02 M solution in H_2O, 30 mol%). DMSO (0.2 mL) was added, the mixture was sonicated for 10 s, and nitrogen was bubbled through the resulting solution for 30 s. This solution was then added to the first vial, additional DMSO (0.5 mL) was added, nitrogen was bubbled through the reaction for 20 s, and the reaction was stirred at room temperature under nitrogen. After 4 h, an aliquot of the reaction was diluted with HPLC grade MeCN and the reaction was analyzed with LC-MS to confirm reaction completion. The reaction was filtered and purified with preparative HPLC (Method C). Product fractions were pooled and concentrated at room temperature (to remove MeOH), and then lyophilized overnight to give **31c** as a colorless oil (7 mg, 58%). LC/MS t_R = 5.66 min (Characterization Method C); m/z = 1794.20 (M + H^+); 1792.05 (M - H^+); 1837.15 (formate adduct).

Protected bivalent ligand AY77-PEG-cilengitide 31d

To an oven dried 5 mL vial with stir bar was added azide **29d** (6.5 mg, 4.4 µmol) and cilengitide-alkyne **5** (3.5 mg, 4.0 µmol). In a separate oven dried 5 mL vial was added sodium ascorbate (2.3 mg, 12 µmol), TBTA (3 mg, 6 µmol), and $CuSO_4$ (0.06 mL of a 0.02 M solution in H_2O, 1.2 µmol, 30 mol%). DMSO (0.4 mL) was added, the mixture was sonicated for 10 sec, and nitrogen gas was bubbled through the resulting solution for 30 s. This solution was then added to the first vial, additional DMSO (0.5 mL) was added, nitrogen was bubbled through the reaction for 20 sec, and the reaction was stirred at room temperature under nitrogen. After 1 h, an aliquot of the reaction was diluted with HPLC grade MeCN and the reaction was analyzed with LC/MS to confirm reaction completion. The reaction was filtered, and then purified with preparative HPLC (Method C). Product fractions were pooled and concentrated at room temperature (to remove MeOH), and then lyophilized overnight to give **31d** as a colorless oil (5.1 mg, 54%). LC/MS t_R = 5.34 min (Characterization Method C**)**; m/z = 1183.80 [(M + H^+/2)].



Bivalent ligand AY77-PEG-cilengitide 32b

To a 5 mL vial was added **31b** (3.5 mg, 2.0 µmol) and a stir bar. The vial was purged with nitrogen and then thioanisole (10 µL), ethanedithiol (6 µL), anisole (4 µL), and TFA (0.36 mL) were added consecutively. The reaction was stirred under nitrogen for 45 min. A drop of the reaction was concentrated to give a crude colorless oil that was dissolved in HPLC grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated under reduced pressure (with base trap attached) in cold water to give a crude yellow oil that was dissolved in DMSO (0.75 mL), filtered, and purified with preparative HPLC (Method E). Product fractions were pooled, concentrated under reduced pressure in cold water (to remove MeOH), and lyophilized overnight to give **32b** as a colorless oil (2.6 mg, 91%). LC/MS t_R = 3.04 min (Characterization Method C); m/z = 1397.85 (M + H^+); 699.20 [(M + H^+/2)]; m/z = 1394.85 (M – H^+); HRMS calcd. for $C_{65}H_{101}N_{15}O_{19}$(M + H^+) 1396.7471, found 1396.7440.

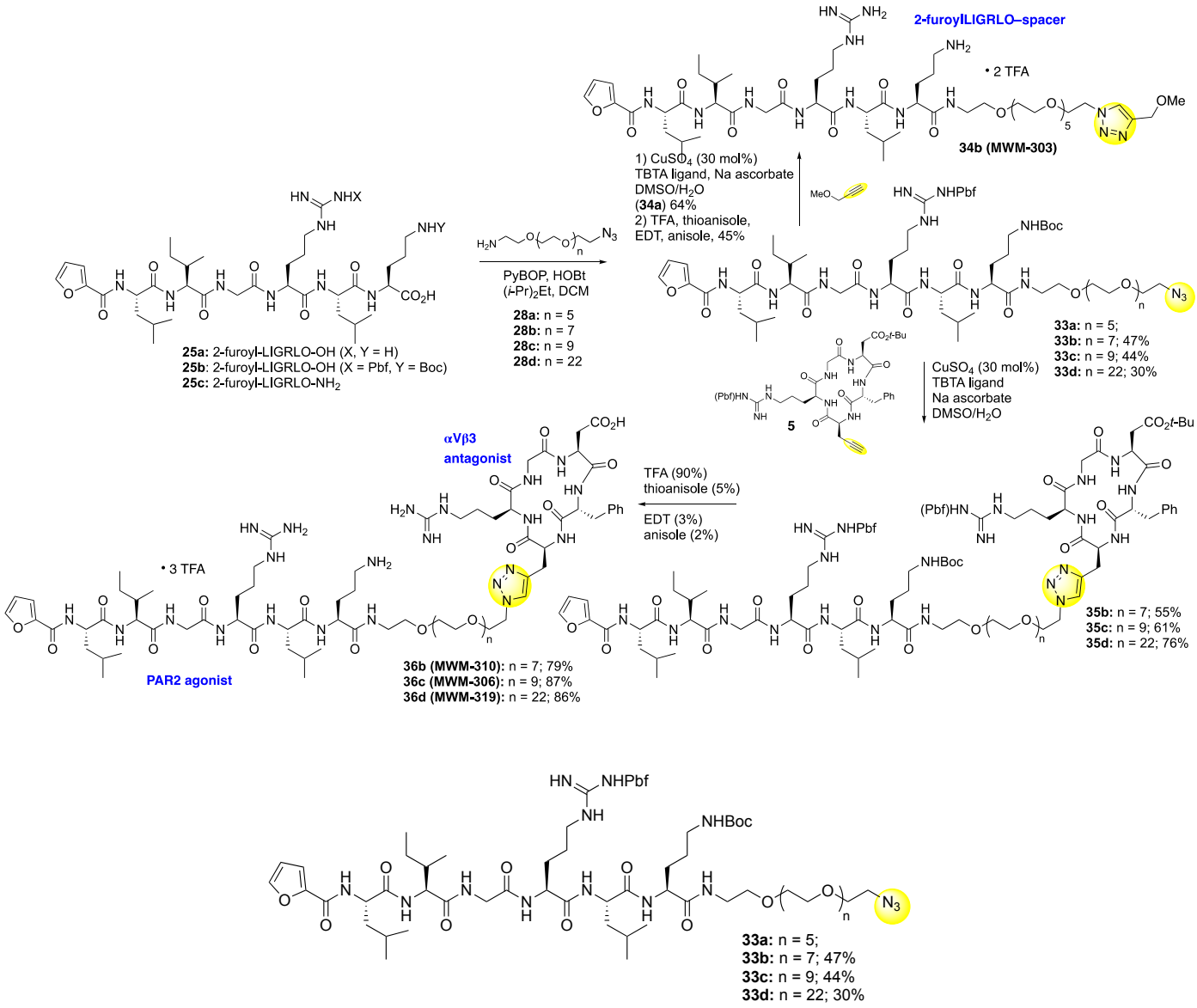
Bivalent ligand AY77-PEG-cilengitide 32c

To a 5 mL vial was added **31c** (3.5 mg, 1.9 µmol). A stir bar was added and the vial was purged with nitrogen. Thioanisole (10 µL), ethanedithiol (6 µL), anisole (4 µL), and TFA (0.36 mL) were added consecutively and the reaction was stirred under nitrogen for 45 min. A drop of the reaction was concentrated to give a crude colorless oil that was dissolved in HPLC grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated under reduced pressure (with base trap attached) in cold water to give a crude oil that was dissolved in DMSO (0.75 mL), filtered, and purified with preparative HPLC (Method E). Product fractions were pooled, concentrated under reduced pressure in cold water (to remove MeOH), and lyophilized overnight to give **32c** as a colorless oil (2.4 mg, 83%). LC/MS t_R = 3.08 min (Characterization Method C); m/z = 1485.90 (M + H^+); 743.30 [(M + H^+/2)]; 1482.85 (M - H^+); HRMS calcd. for $C_{69}H_{109}N_{15}O_{21}Na$ (M + Na^+) 1506.7815, found 1506.7780.

Bivalent ligand AY77-PEG-cilengitide 32d

To a 5 mL vial was added **31d** (4 mg, 1.7 µmol). A stir bar was added and the vial was purged with nitrogen. Thioanisole (10 µL), ethanedithiol (6 µL), anisole (4 µL), and TFA (0.36 mL) were added consecutively and the reaction was stirred under nitrogen for 45 min. A drop of the reaction was concentrated under reduced pressure to give a crude oil that was dissolved in HPLC grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated under reduced pressure (with base trap attached) in cold water to give a crude yellow oil that was dissolved in DMSO (0.75 mL), filtered, and purified with preparative HPLC (Method E). Product fractions were pooled, concentrated under reduced pressure in cold water (to remove MeOH), and lyophilized overnight to give **32d** as a colorless oil (2.6 mg, 75%). LC/MS t_R = 3.09 min (Characterization Method C); m/z = 1029.55 [(M + H$^+$/2)]; HRMS (ESI-TOF) calcd. for $C_{95}H_{161}N_{15}O_{34}$ (M – H$^+$) 2055.1288, found 2055.1031.

Scheme 5. Assembly of heterobivalent ligands featuring cilengitide and 2-furoyl-LIGRLO-NH$_2$



2-furoyl-LIGRLO-PEG-azide 33b

To an oven dried 20 mL vial under nitrogen was added 2-furoyl-2-LIGRLO-OH (**25a**) (15.0 mg, 13.3 µmol), 26-azido-3,6,9,12,15,18,21,24-octaoxahexacosan-1-amine (**28b**) (7.5 mg, 17.1 µmol), PyBOP (10.0 mg, 19.2 µmol), and HOBt (3.0 mg, 19.6 µmol). Anhydrous DCM (1 mL) and DIPEA (0.29 mL of a 0.05 M solution in DCM, 13.2 µmol) were added and the reaction was stirred for 90 min under N_2. An aliquot from the reaction was concentrated to give an oil that was dissolved in LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated in lukewarm water to give crude product that was dissolved in DMSO (1.5 mL), filtered, and purified with

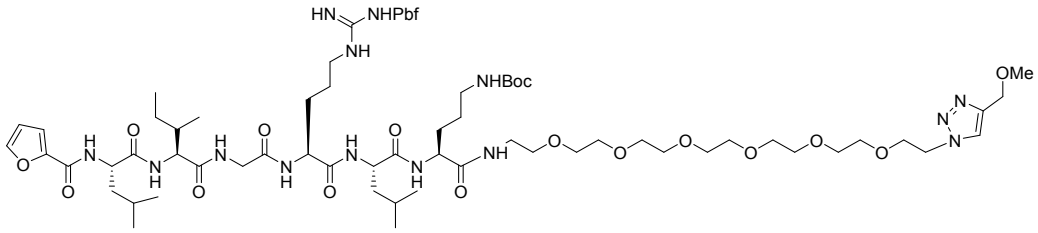
preparative HPLC (Method D). Product fractions were pooled, concentrated under reduced pressure in lukewarm water (to remove MeCN), and lyophilized overnight to give **33b** as a colorless oil (9 mg, 47%). LC/MS t_R = 5.76 min (Characterization Method C); m/z = 1552.05 ($M + H^+$), m/z = 1550.00 ($M - H^+$).

2-furoyl-LIGRLO-PEG-azide 33c

To an oven dried 5 mL vial under nitrogen was added 2-furyl-2-LIGRLO-OH (**25a**) (12.0 mg, 10.6 µmol), 32-azido-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontan-1-amine (**28c**) (8.5 mg, 16.1 µmol), PyBOP (8.2 mg, 15.9 µmol), and HOBt (2.4 mg, 15.9 µmol). Anhydrous DCM (2 mL) and DIPEA (0.23 mL of a 0.05 M solution in DCM, 10.5 µmol) were added and the reaction was stirred for 2 h under N_2. An aliquot from the reaction was concentrated to give an oil that was dissolved in LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated in lukewarm water to give a yellow oil that was dissolved in DMSO (1.5 mL), filtered, and purified with preparative HPLC (Method D). Product fractions were pooled, concentrated in lukewarm water (to remove MeCN), and lyophilized overnight to give **33c** as a colorless oil (7.5 mg, 44%). LC/MS t_R = 5.71 min (Characterization Method C); m/z = 1641.10 ($M + H^+$), m/z = 1639.00 ($M - H^+$).

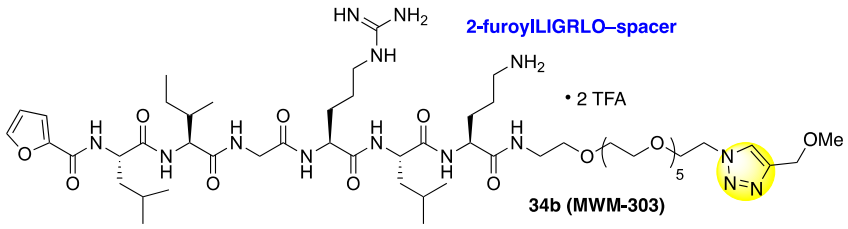
2-furoyl-LIGRLO-PEG-azide 33d

To an oven dried 20 mL vial with stir bar and under N_2 was added 2-furoyl-2-LIGRLO-OH (**25a**) (12 mg, 10.6 µmol), 71-azido-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69-tricosaoxahenheptacontan-1-amine (**28d**) (14 mg, 12.7 µmol), PyBOP (11 mg, 21.2 µmol), and HOBt (2.4 mg, 15.9 µmol). Anhydrous DCM (1 mL) and DIPEA (0.24 mL of a 0.05 M solution in DCM, 11 µmol) were added and the reaction was stirred for 90 min. under N_2. An aliquot from the reaction was concentrated to give an oil that was dissolved in LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated to give a crude yellow oil that was dissolved in DMSO (1.5 mL), filtered, and purified with preparative HPLC (Method B). Product fractions were pooled, concentrated in lukewarm water (to remove MeOH), and lyophilized overnight to give **33d** as a colorless oil (7 mg, 30%). LC/MS t_R = 5.49 min (Characterization Method C); m/z = 1107.10 [($M + H^+$/2)], m/z = 738.5 [($M + H^+$/3)].



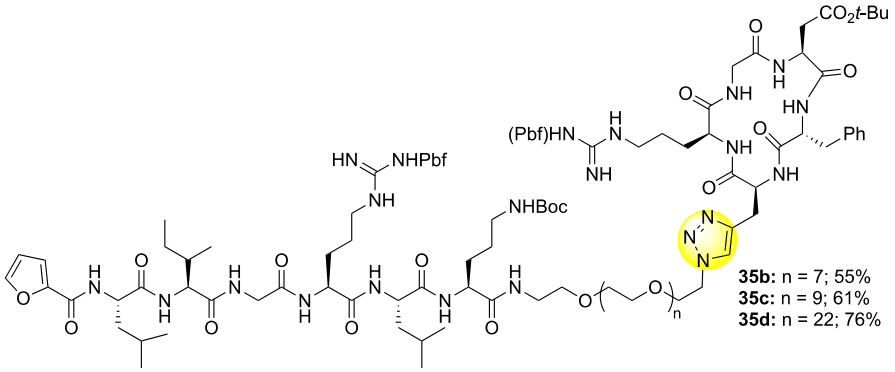
Protected 2-furoyl-LIGRLO-PEG-triazole 34a

To a 5 mL vial was added azide **33b** (5 mg, 3.4 µmol), propargyl methyl ether (0.29 mL of a 0.02 M solution in DMSO, 5.8 µmol), and stir bar. In a second 5 mL vial was added sodium ascorbate (2.8 mg, 14.0 µmol), TBTA (2.8 mg, 3.8 µmol), and $CuSO_4$ (20 mol%, 35 µL of a 0.02 M solution in H_2O, 0.68 µmol). Additional DMSO (0.4 mL) was added, the mixture sonicated for 20 s, and nitrogen was bubbled through the resulting solution for 20 s. This solution was then added to the first vial, nitrogen was bubbled through the reaction for 20 s, and the reaction was stirred for 1 h under nitrogen. A drop of the reaction was diluted with LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was filtered and purified with preparative HPLC (Method D). Product containing fractions were pooled together, concentrated (to remove MeCN), and then lyophilized overnight to give **34a** as a colorless oil (3.4 mg, 64%). LC/MS t_R = 5.39 min (Method C); m/z = 1535.00 ($M + H^+$), m/z = 1532 ($M - H^+$); HRMS calcd. for $C_{72}H_{120}N_{14}O_{20}SNa$ ($M + Na^+$) 1555.8416, found 1555.8400.



2-Furoyl-LIGRLO-PEG-triazole 34b

To an oven dried 5 mL vial was added # (3.5 mg, 2.2 μmol) and stir bar. The vial was purged with N_2 and thioanisole (10 μL), ethanedithiol (6 μL), anisole (4 μL), and TFA (0.360 mL) were added consecutively. The reaction was stirred under nitrogen for 30 min. An aliquot from the reaction was concentrated under reduced pressure to give a crude yellow oil that was dissolved in LCMS grade MeCN and analyzed with LC/MS to confirm reaction completion. The reaction was concentrated under reduced pressure in cold water to give a crude yellow oil that was dissolved in DMSO (750 μL), syringe filtered (0.2 μm), and purified with preparative HPLC (Method E). Product fractions were pooled, concentrated under reduced pressure in cold water (to remove MeOH), and lyophilized overnight to give # (1.4 mg) as a colorless oil in 45% yield. LC/MS t_R = 3.53 min (Characterization Method B); m/z = 1181.70 ($M + H^+$); 1179.70 ($M - H^+$). HRMS calcd. for $C_{54}H_{96}N_{14}O_{15}$



Protected 2-furoyl-LIGRLO-PEG-cilengitide analog 35b

To a 20 mL vial was added azide **33b** (9 mg, 5.8 μmol), cilengitide-alkyne **5** (4.5 mg, 5.1 μmol), and a stir bar. In a separate 5 mL vial was added sodium ascorbate (3.0 mg, 15 μmol), TBTA (5.2 mg, 9.8 μmol), and $CuSO_4$ (80 μL of a 0.02 M solution in H_2O, 1.5 μmol, 30 mol%). DMSO (0.5 mL) was added, the mixture was sonicated for 10 s and N_2 was bubbled through the resulting solution for 30 seconds. This solution was then added to the first vial, additional DMSO (1.0 mL) was added, nitrogen was bubbled through the reaction for 20 s, and the reaction was stirred for 1 h at room temperature under nitrogen. An aliquot from the reaction was diluted with LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was filtered (0.2 μm) and then purified with preparative HPLC (Method C). Product fractions were pooled together and concentrated at room temperature (to remove MeOH), and then lyophilized overnight to give **35b** as a colorless oil (6.8 mg, 55%). LC/MS t_R = 6.02 min (Characterization Method C); m/z = 1216.30 [$(M + H^+)/2$], m/z = 1214.15 [$(M - H^+)/2$].

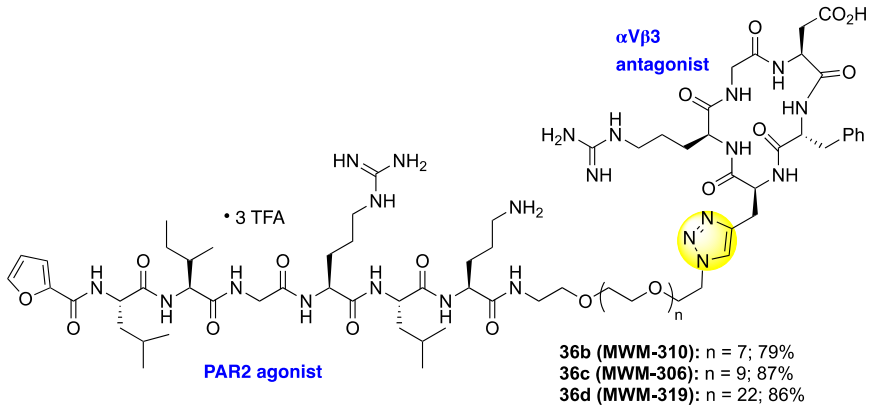
Protected 2-furoyl-LIGRLO-PEG-cilengitide analog 35c

To a 20 mL vial was added azide **33c** (7.5 mg, 4.5 μmol), the cilengitide-alkyne **5** (4.2 mg, 4.8 μmol), and a stir bar. In a separate 5 mL vial was added sodium ascorbate (2.7 mg, 14 μmol), TBTA (4.8 mg, 9.2 μmol), and $CuSO_4$ (70 μL of a 0.02 M solution in H_2O, 30 mol%). DMSO (0.2 mL) was added, the mixture was sonicated for 10 s, and nitrogen was bubbled through the resulting solution for 30 s. This solution was then added to the first vial, additional DMSO (0.5 mL) was added, nitrogen was bubbled through the reaction for 20 s, and the reaction was stirred for 2 h under nitrogen. An aliquot of the reaction was diluted with LC-MS grade MeCN and the reaction was analyzed with LC-MS to confirm completion. The reaction was passed through a syringe filter (0.2 μm filter) and then purified with preparative HPLC (Method C). Product fractions were pooled and concentrated at room temperature (to remove MeOH), and then lyophilized overnight to give **35c** as a colorless oil (7 mg, 61%). LC/MS t_R = 6.01 min (Characterization Method C); m/z = 1260.35 [$(M + H^+)/2$], m/z = 1258.25 [$(M - H^+)/2$].

Protected 2-furoyl-LIGRLO-PEG-cilengitide analog 35d

To a 20 mL vial was added azide **33d** (8.3 mg, 3.8 μmol), cilengitide-alkyne **5** (3 mg, 3.4 μmol), and a stir bar. In a separate 5 mL vial was added sodium ascorbate (1.7 mg, 8.5 μmol), TBTA (2.6 mg, 4.9 μmol), and $CuSO_4$ (50 μL of a 0.02 M solution in H_2O, 1.5 μmol, 30 mol%). DMSO (0.5 mL) was added, the mixture was sonicated for 10 s, and nitrogen was bubbled through the resulting solution for 30 s. This solution was then added to the first vial, additional DMSO (1.0 mL) was added, nitrogen was bubbled through the reaction for 20 s, and the reaction was stirred for 1 h at room temperature under nitrogen. An aliquot of the reaction was diluted with LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was filtered (0.2 μm filter) and then purified with preparative HPLC (Method B). Product fractions were pooled and concentrated at room temperature (to remove MeOH), and then

lyophilized overnight to give **35d** as a colorless oil (8 mg, 76%). LC/MS t_R = 5.75 min (Characterization Method C); m/z = 1546.75 [(M + H^+)/2], m/z = 1544.70 [(M - H^+)/2].



Bivalent ligand 2-furoyl-LIGRLO-PEG-cilengitide analog 36b

To an oven dried 5 mL vial was added **35b** (3.5 mg, 1.4 µmol) and a stir bar. The vial was purged with nitrogen and thioanisole (10 µL), ethanedithiol (6 µL), anisole (4 µL), and TFA (0.360 mL) were added consecutively. The reaction was stirred under nitrogen for 30 min. An aliquot from the reaction was concentrated under reduced pressure to give a yellow oil that was dissolved in LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated under reduced pressure in cold water to give a yellow oil that was dissolved in DMSO (0.75 mL), syringe filtered (0.2 µm), and purified with preparative HPLC (Method E). Product fractions were pooled, concentrated under reduced pressure in cold water (to remove MeOH), and lyophilized overnight to give **36b** as a colorless oil (2.0 mg, 79%). LC/MS t_R = 3.43 min (Characterization Method B); m/z = 1768.00 (M - H^+). HRMS calcd. for $C_{80}H_{132}N_{22}O_{23}$ (M + H^+) 1769.9908, found 1769.9859.

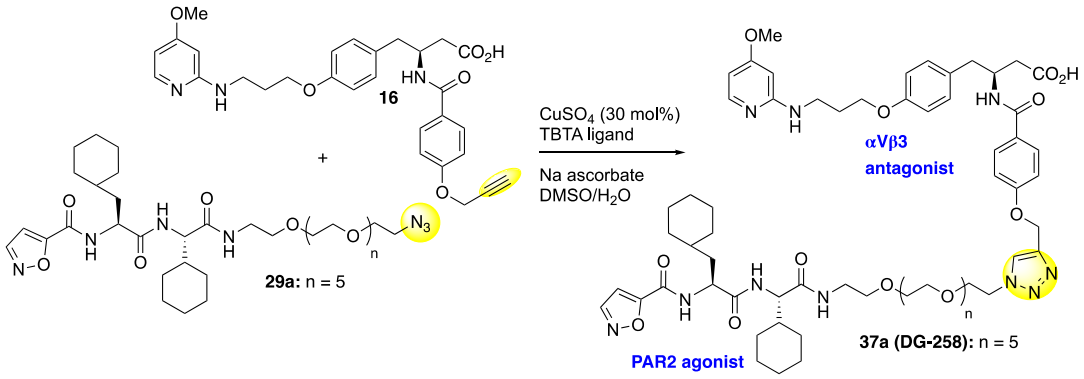
Bivalent ligand 2-furoyl-LIGRLO-PEG-cilengitide analog 36c

To an oven dried 5 mL vial was added **35c** (3.5 mg, 1.4 µmol) and a stir bar. The vial was purged with nitrogen for 5 min and thioanisole (10 µL), ethanedithiol (6 µL), anisole (4 µL), and TFA (0.36 mL) were added consecutively and the reaction was stirred under nitrogen for 45 min. An aliquot of the reaction was concentrated under reduced pressure to give a yellow oil that was dissolved in LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated under reduced pressure (with base trap attached) in cold water to give a crude yellow oil that was dissolved in DMSO (750 µL), syringe filtered (0.2 µm), and purified with preparative HPLC (Method E). Product fractions were pooled, concentrated under reduced pressure in cold water (to remove MeOH), and lyophilized overnight to give **36c** as a colorless oil (2.2 mg, 86%). LC/MS t_R = 1.64 min (Characterization Method C); m/z = 929.95 [(M + H^+)/2], m/z = 1857.15 (M - H^+). HRMS calcd. for $C_{84}H_{140}N_{22}O_{25}Na$ (M + Na^+) 1880.0252, found 1880.0236.

Bivalent ligand 2-furoyl-LIGRLO-PEG-cilengitide analog 36d

To an oven dried 5 mL vial was added **35d** (4 mg, 1.3 µmol) and a stir bar. The vial was purged with nitrogen for 5 min., then thioanisole (10 µL), ethanedithiol (6 µL), anisole (4 µL), and TFA (0.36 mL) were added consecutively, and the reaction was stirred under nitrogen for 45 min. An aliquot from the reaction was concentrated to give a yellow oil that was dissolved in LC-MS grade MeCN and analyzed with LC-MS to confirm reaction completion. The reaction was concentrated under reduced pressure (with base trap attached) in cold water to give a yellow oil that was dissolved in DMSO (0.75 mL), syringe filtered (0.2 µm), and purified with preparative HPLC (Method E). Product fractions were pooled, concentrated under reduced pressure in cold water (to remove MeOH), and lyophilized overnight to give **36d** as a colorless oil (2.7 mg, 86%). *Note: This compound demonstrated solubility issues in solvents besides DMSO, as indicated by shouldering of LC-MS peaks unless small quantities were injected.* LC/MS t_R = 2.21 min (Characterization Method C); m/z = 1216.35 [(M + H^+)/2], m/z = 1214.25 [(M - H^+)/2]. HRMS (ESI-TOF) calcd. for $C_{110}H_{192}N_{22}O_{38}$ (M – H^+) 2428.3695, found 2428.3402.

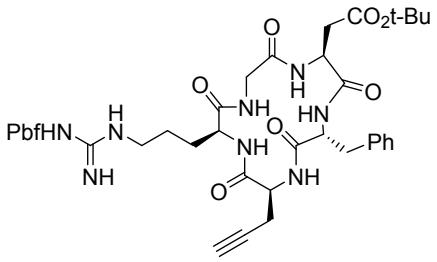
Scheme 6. Assembly of heterobivalent ligands featuring Kessler's ligand and AY77

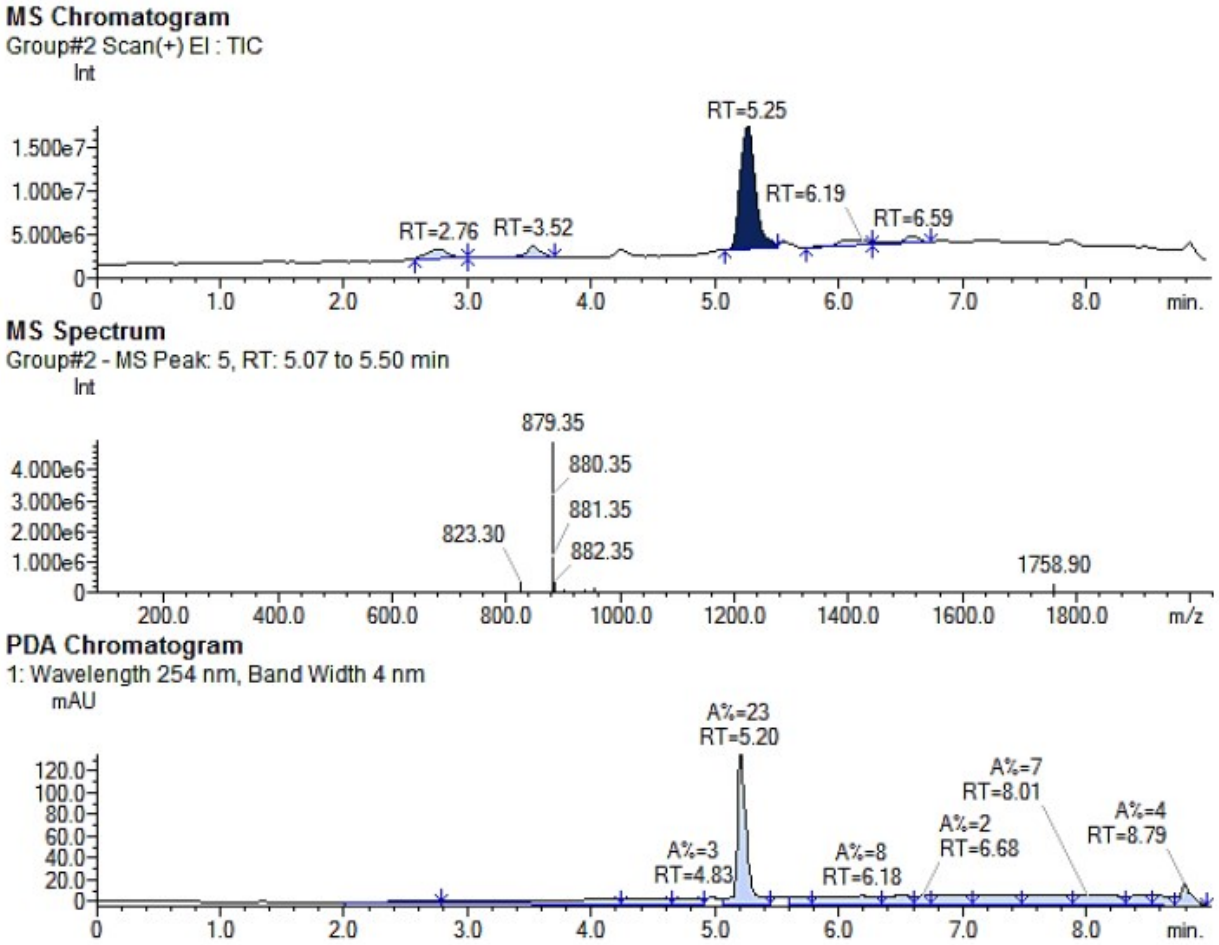


Bivalent ligand AY77-PEG-Kessler antagonist 37a

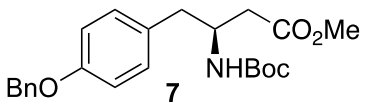
To 4 mL pre-sterilized vial, isooxazole azide **29a** (3.00 mg, 4.07 µmol), alkyne **16** (2.40 mg, 4.64 µmol) and TBTA ligand (0.70 mg, 1.32 µmol) were added with a stir bar, followed by addition of 350 µL DMF. The vial was flushed with nitrogen for 10 min. Sodium ascorbate (1.30 mg, 6.40 µmol) and 0.02 M copper (II) sulfate in H_2O (70 µL, 1.40 µmol) were added to the vial, which was then flushed with nitrogen for 2 min., and the resultant reaction was stirred for 1 h. After 1 h, an aliquot was taken via 25 µL syringe, diluted with HPLC grade MeOH, filtered through a 0.22 µM PTFE syringe filter. LC-MS analysis showed complete consumption of starting material. The reaction was filtered through a 0.22 µM PTFE syringe filter, washed with HPLC grade DMSO, and purified with preparative HPLC (Method B). Product fractions were pooled, concentrated under reduced pressure (to remove MeOH), and lyophilized overnight to give **37a** as a white solid (2.6 mg, 51%). LC/MS t_R = 5.81 min (Characterization Method A); calculated m/z = 1254.48 ($M - H^+$), observed m/z = 1254.60 ($M - H^+$).

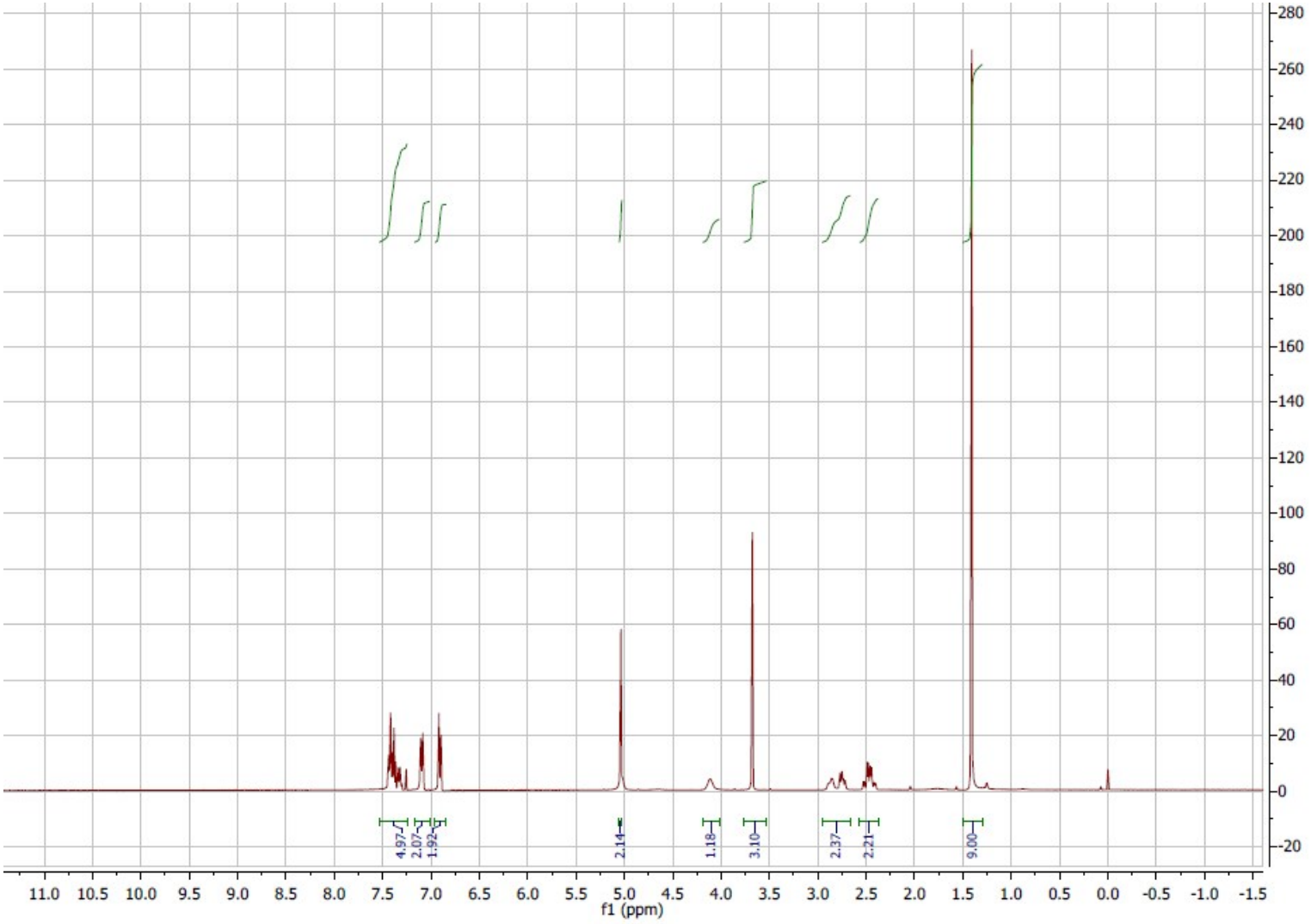
5. NMR and LC-MS Data for Select Compounds



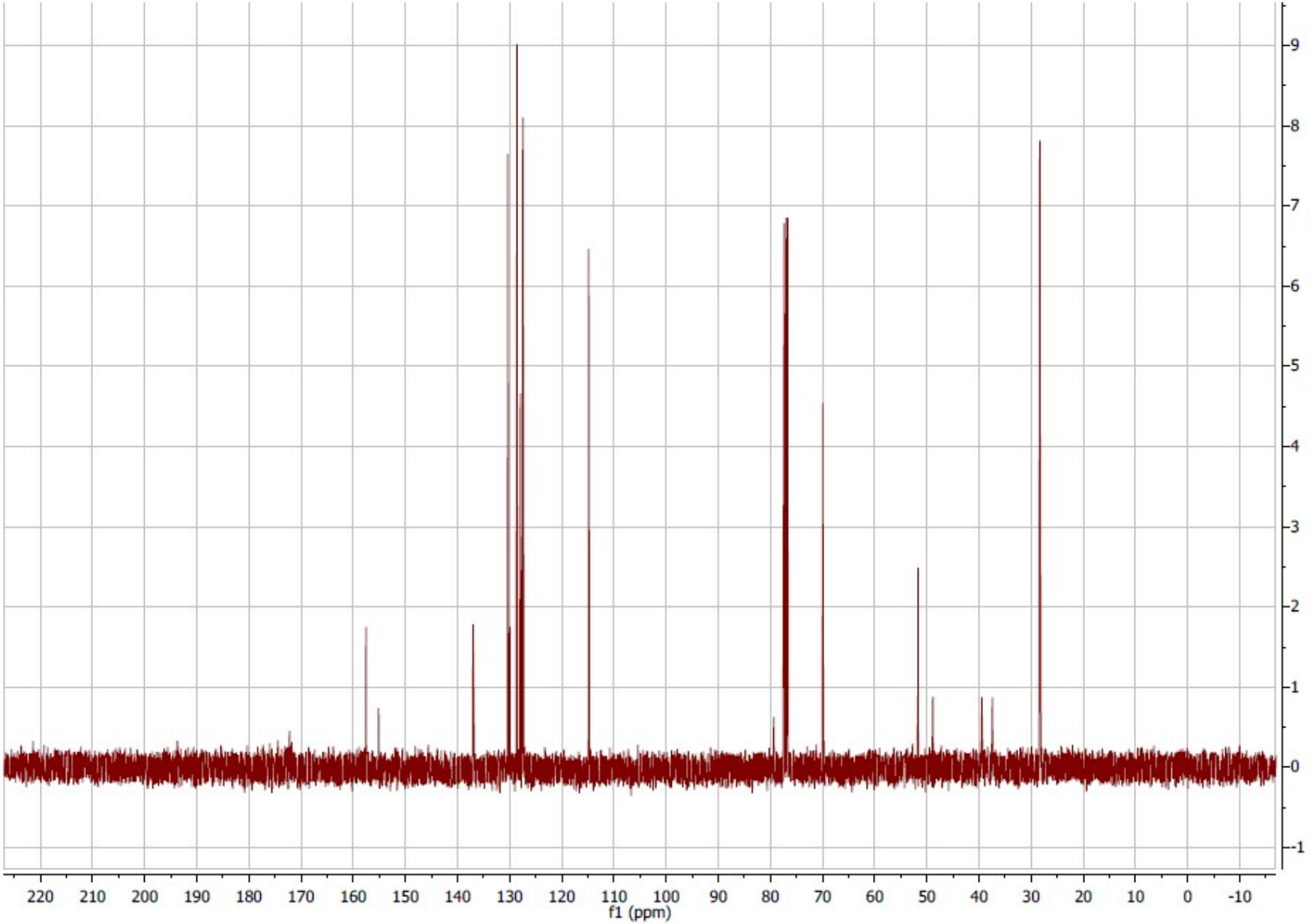


LC/MS of **5** (+ mode)

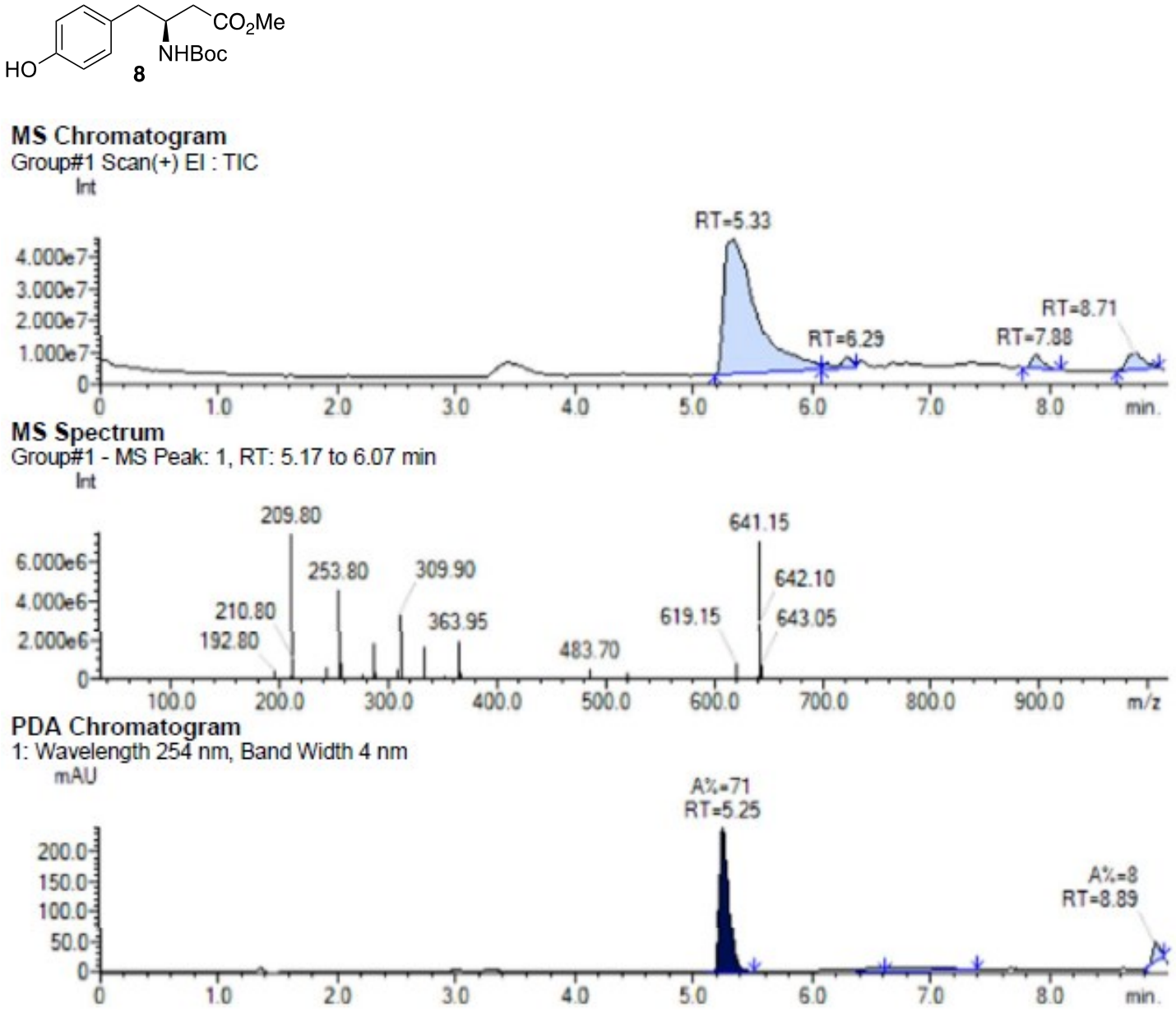




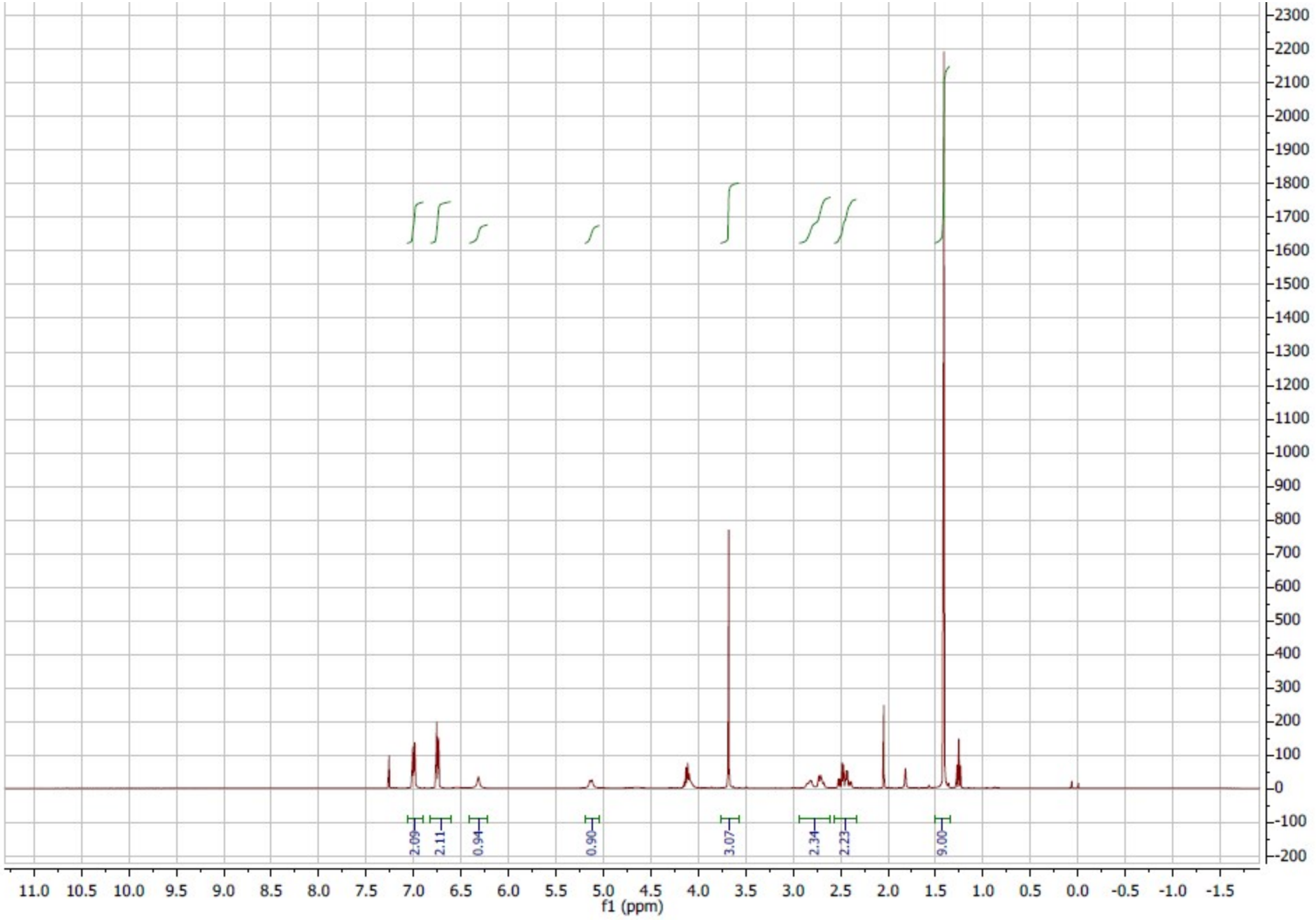
^{1}H NMR of **7** (400 MHz, $CDCl_3$)



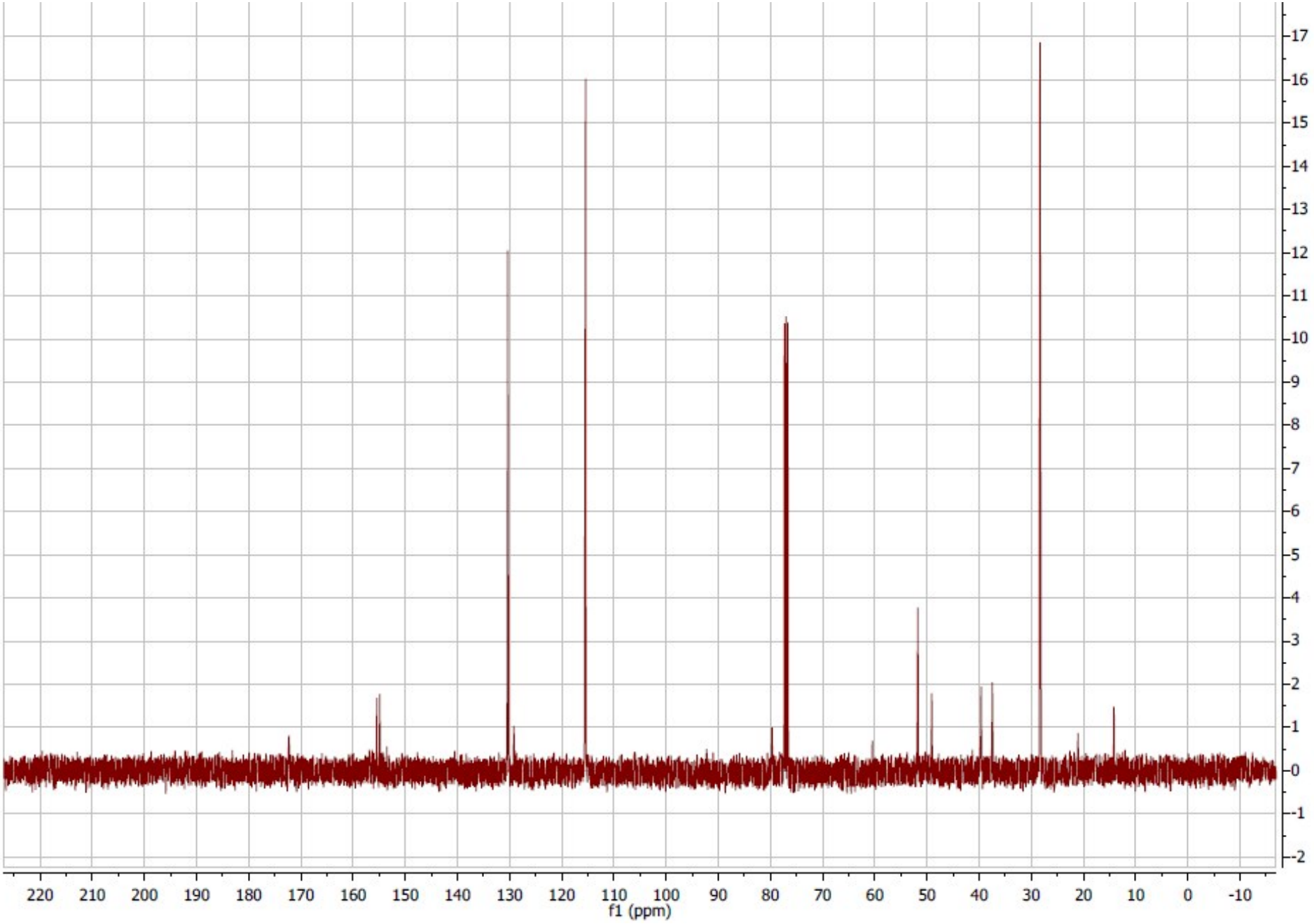
^{13}C NMR of **7** (101 MHz, $CDCl_3$)



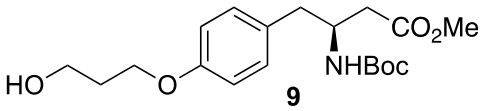
LC/MS of **8** (+ mode)


2.09
2.11
0.94
0.90
3.07
2.34
2.23
9.00
f1 (ppm)

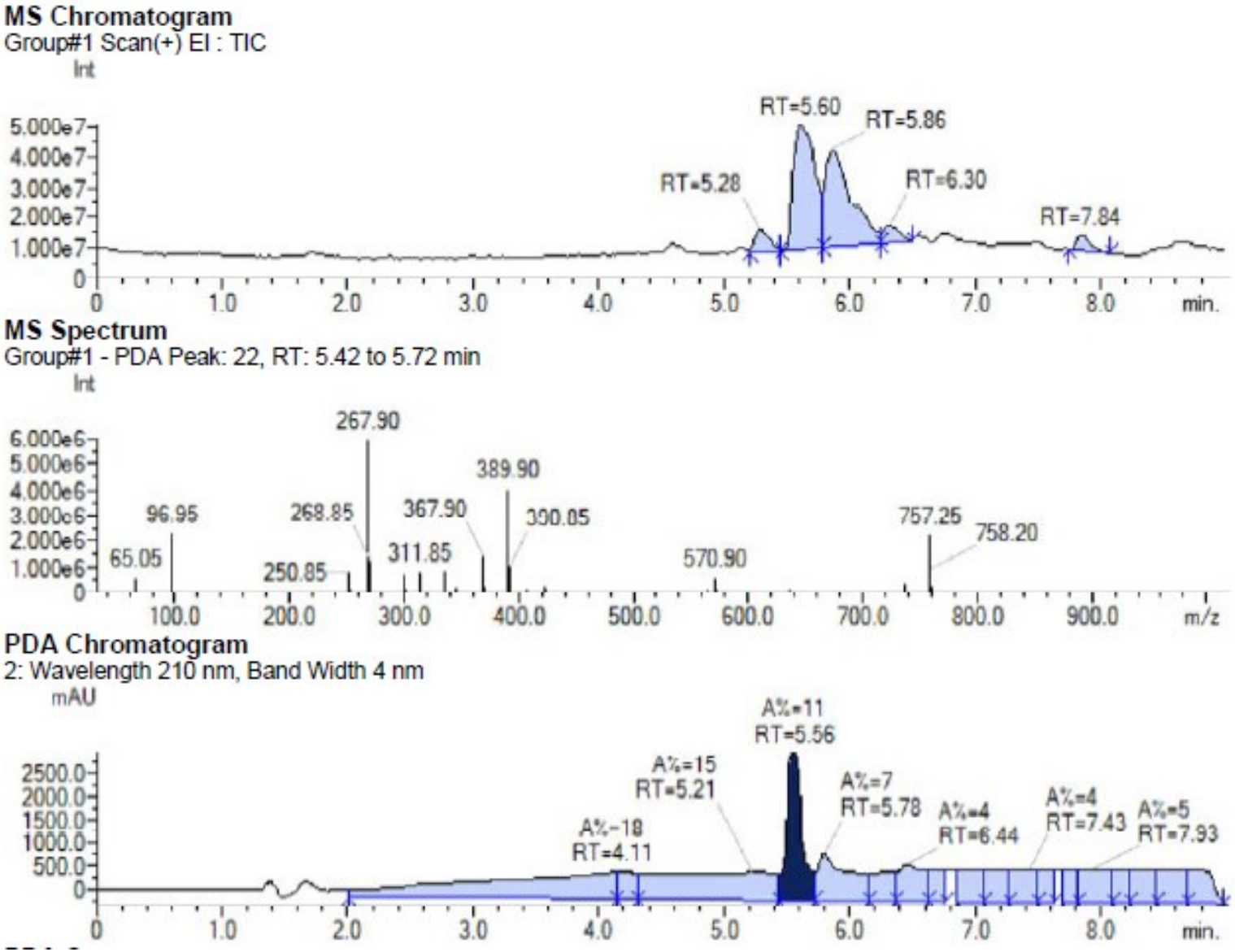
^{1}H NMR of **8** (400 MHz, $CDCl_3$)


17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

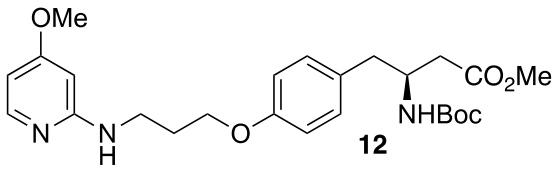
^{13}C NMR of **8** (101 MHz, $CDCl_3$)



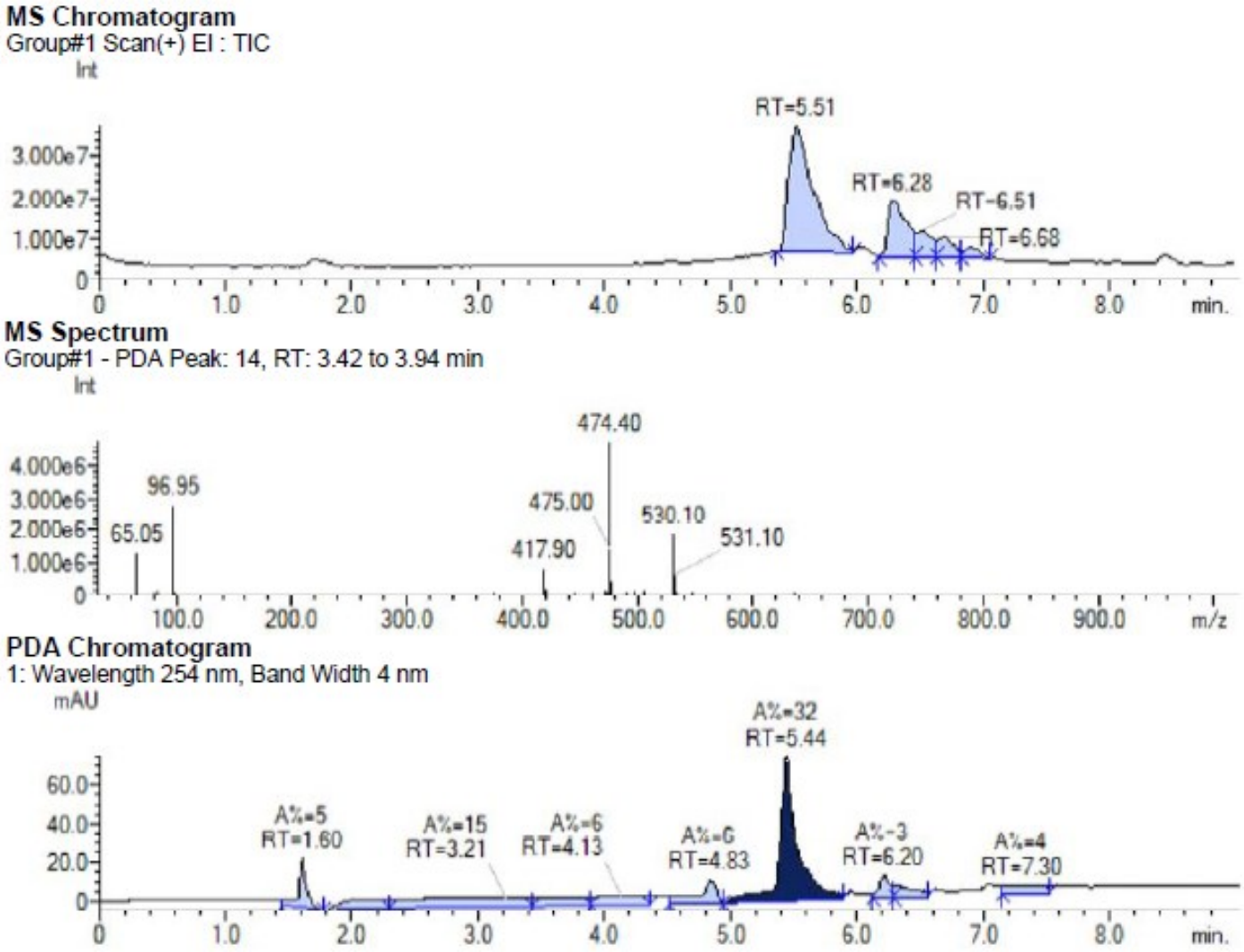
9



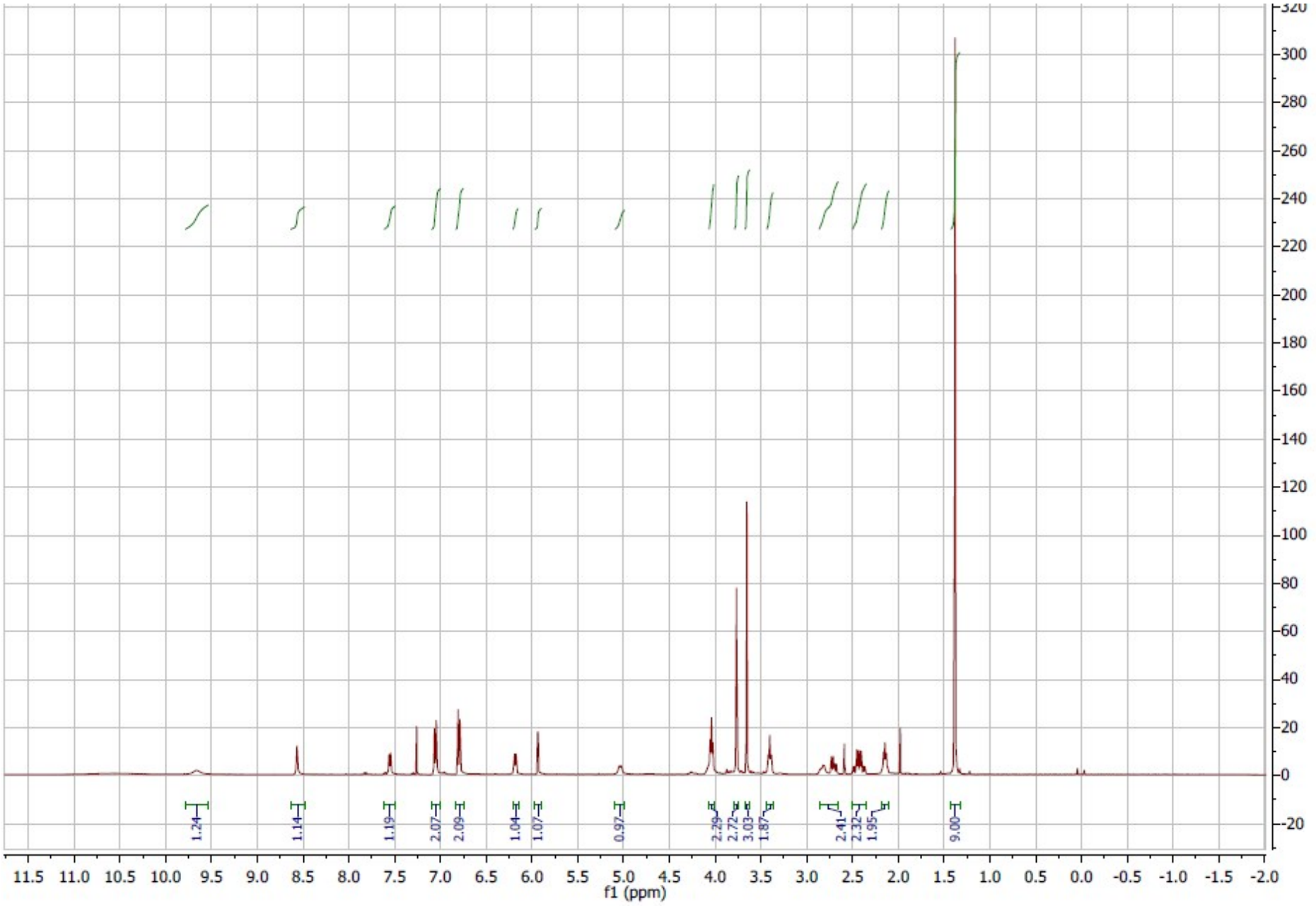
LC/MS of **9** (+ mode)



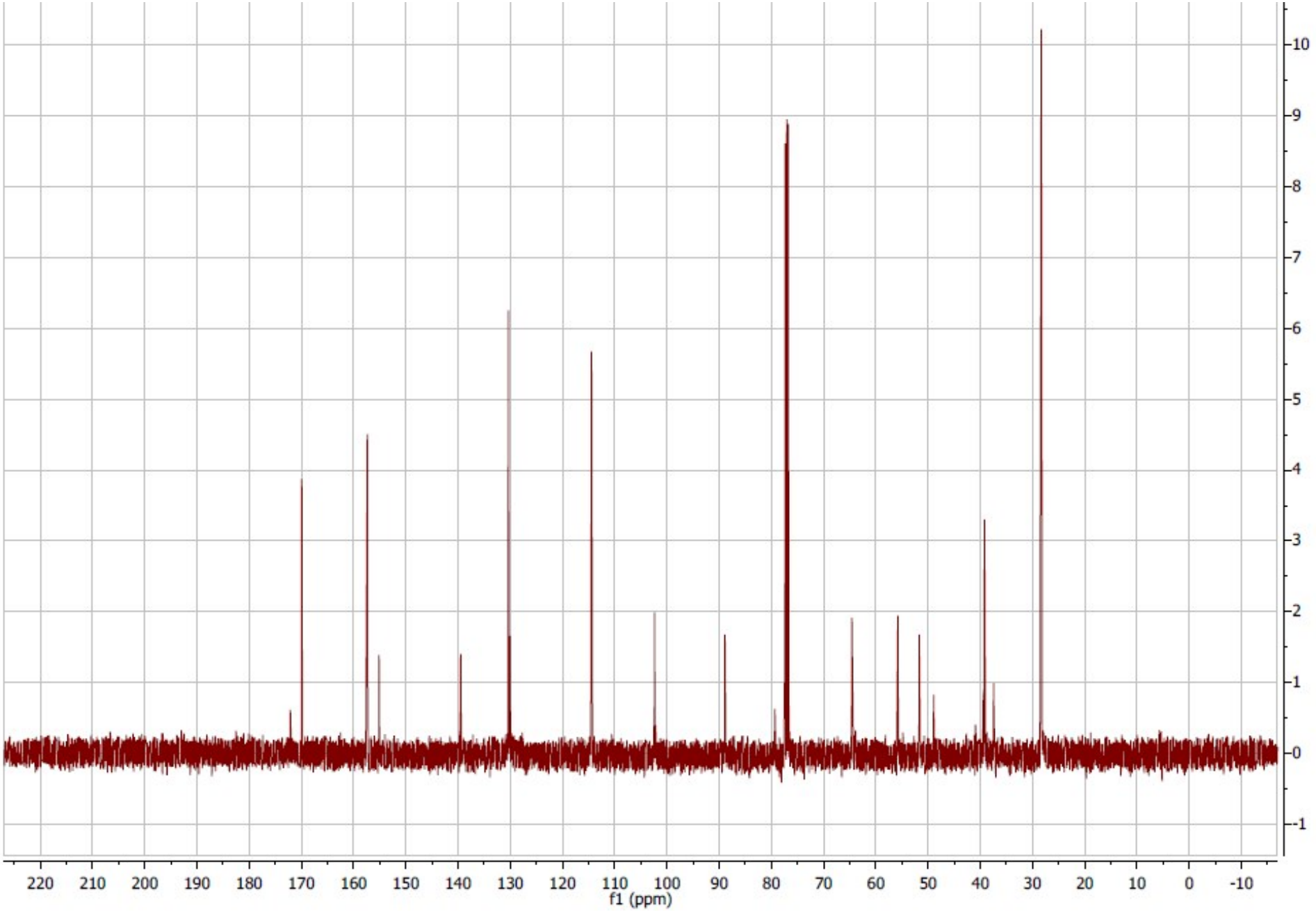
12



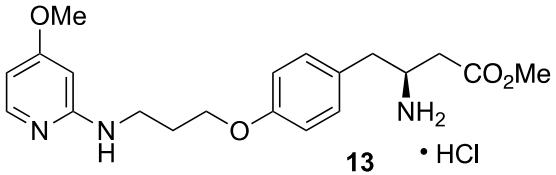
LC/MS of **12** (+ mode)


1.24
1.14
1.19
2.07
2.09
1.04
1.07
0.97
2.29
2.72
3.03
1.87
2.41
2.32
1.95
9.00
f1 (ppm)

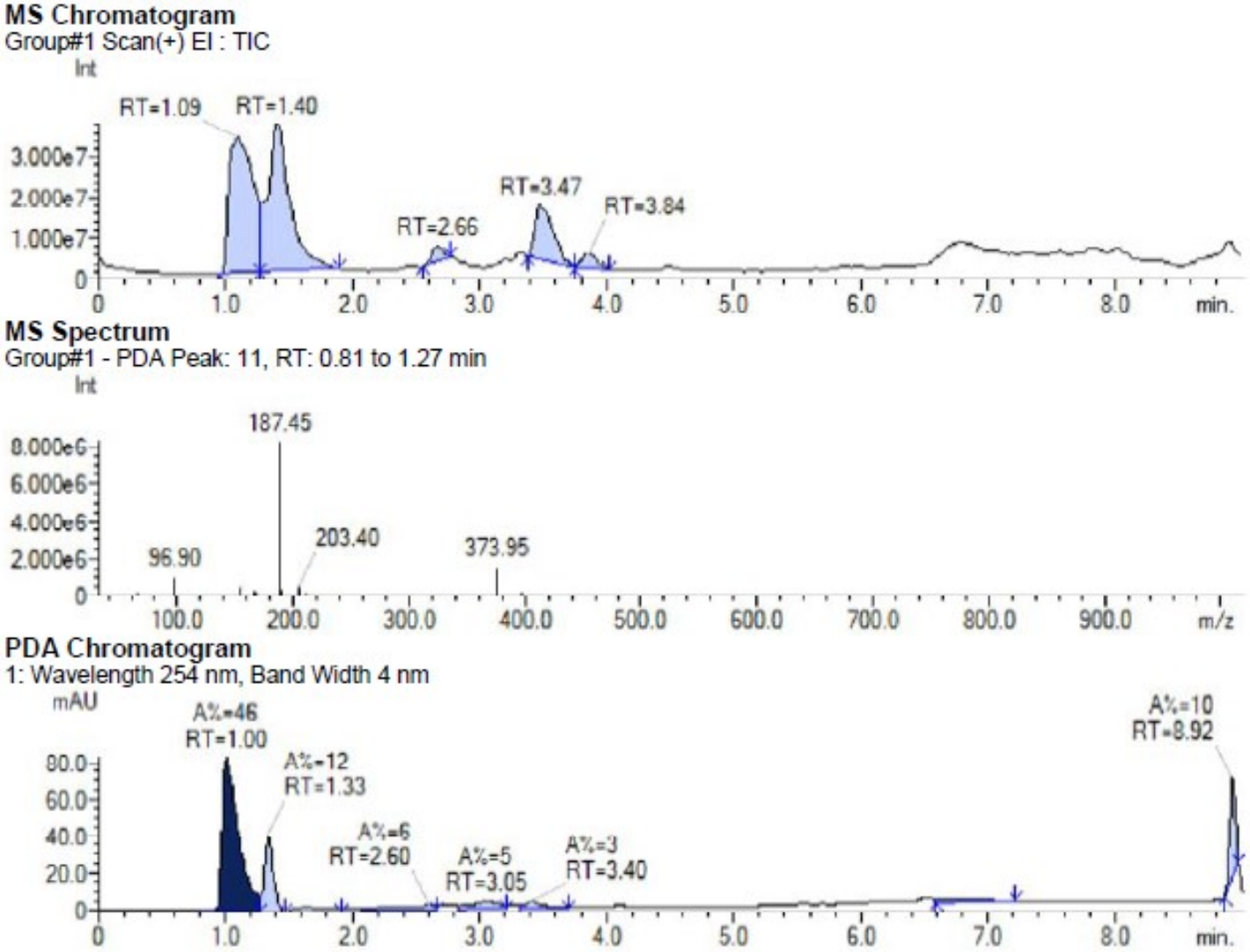
^{1}H NMR of **12** (400 MHz, $CDCl_3$)


f1 (ppm)

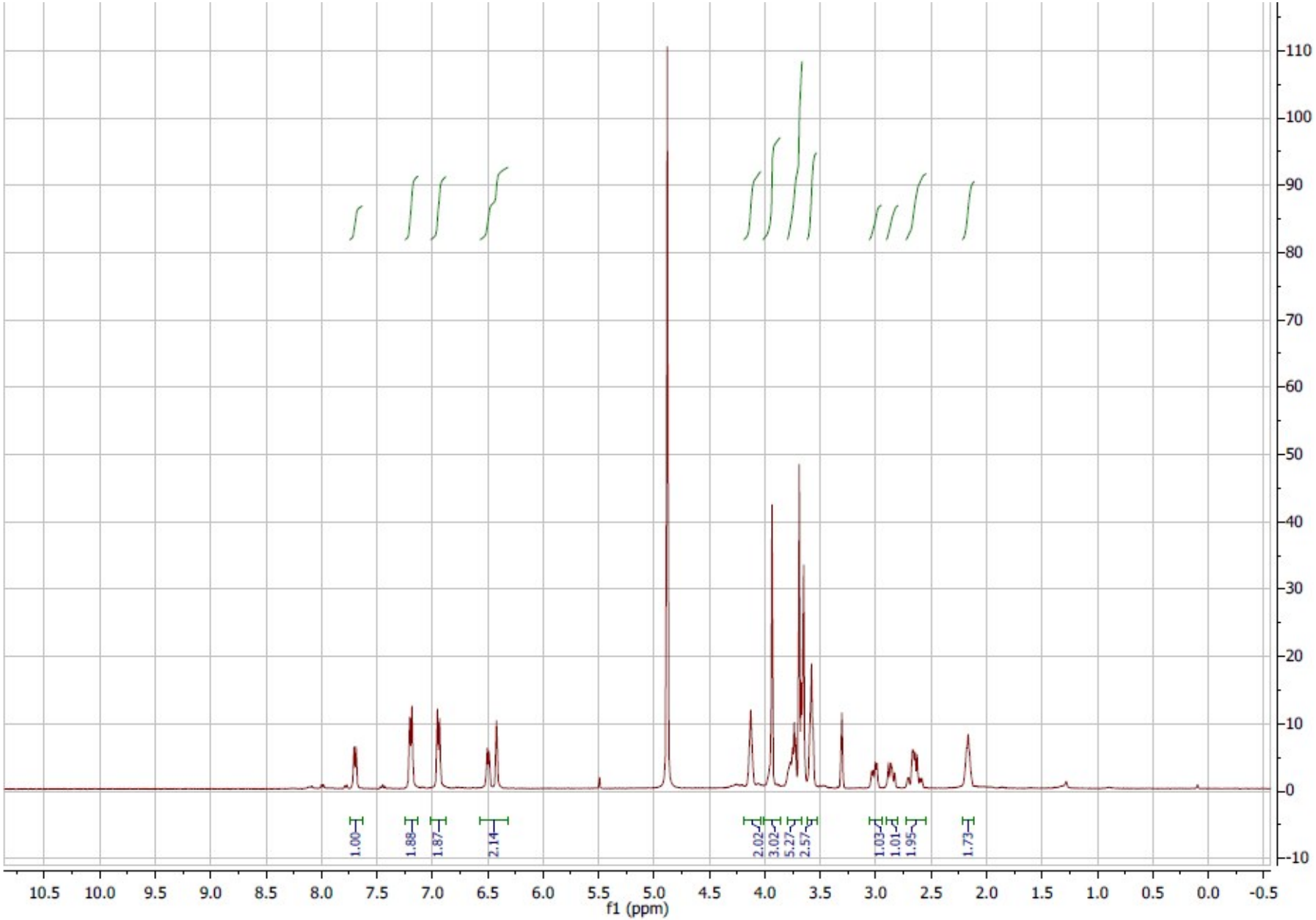
^{13}C NMR of **12** (101 MHz, $CDCl_3$)



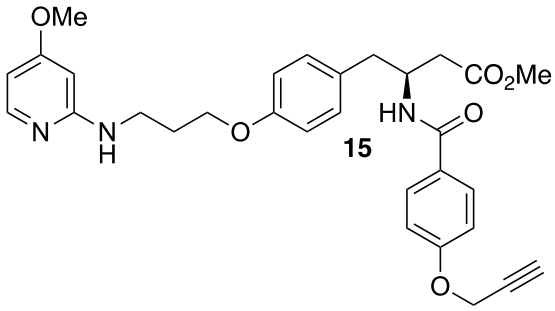
13 • HCl

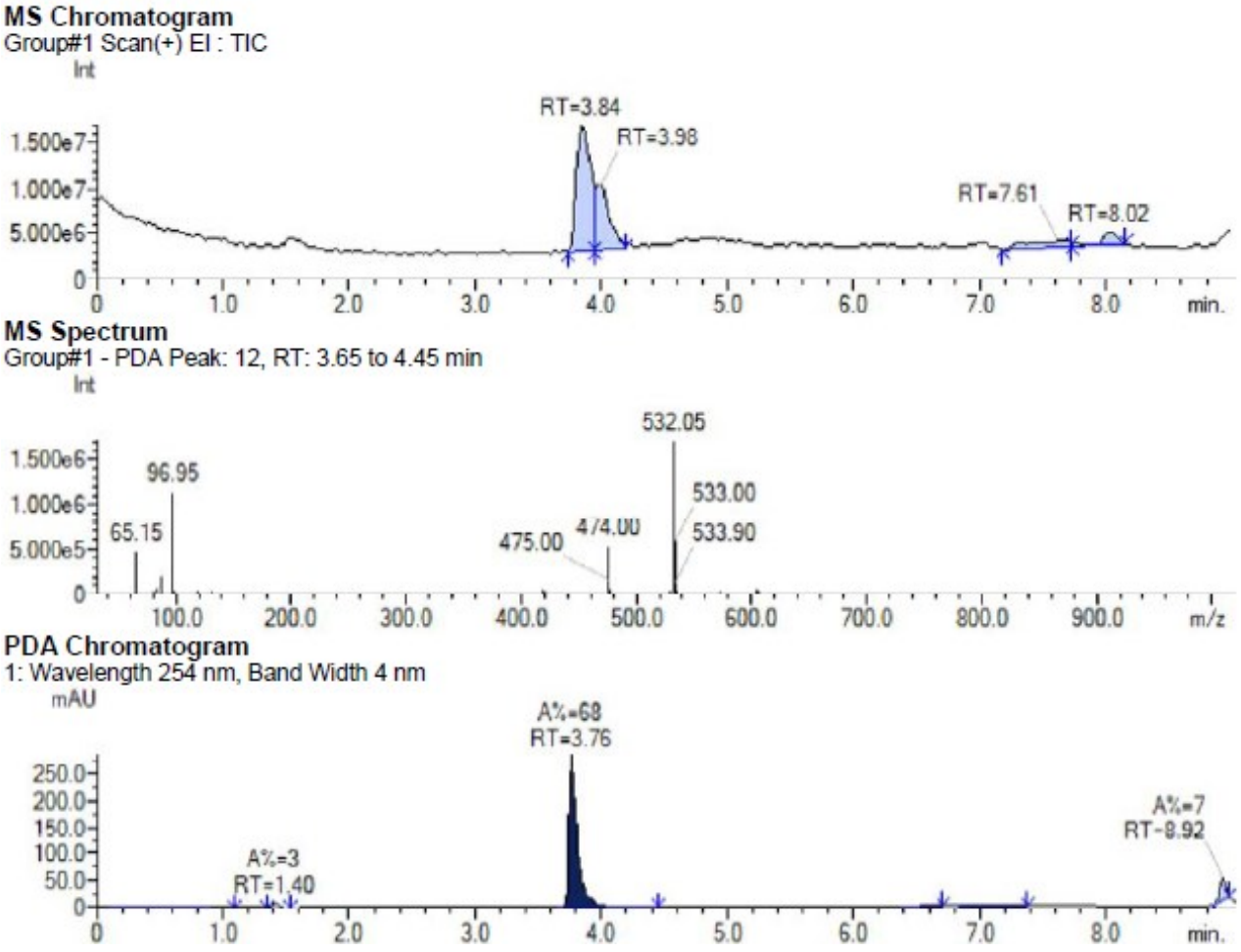


LC/MS of **13** (+ mode)

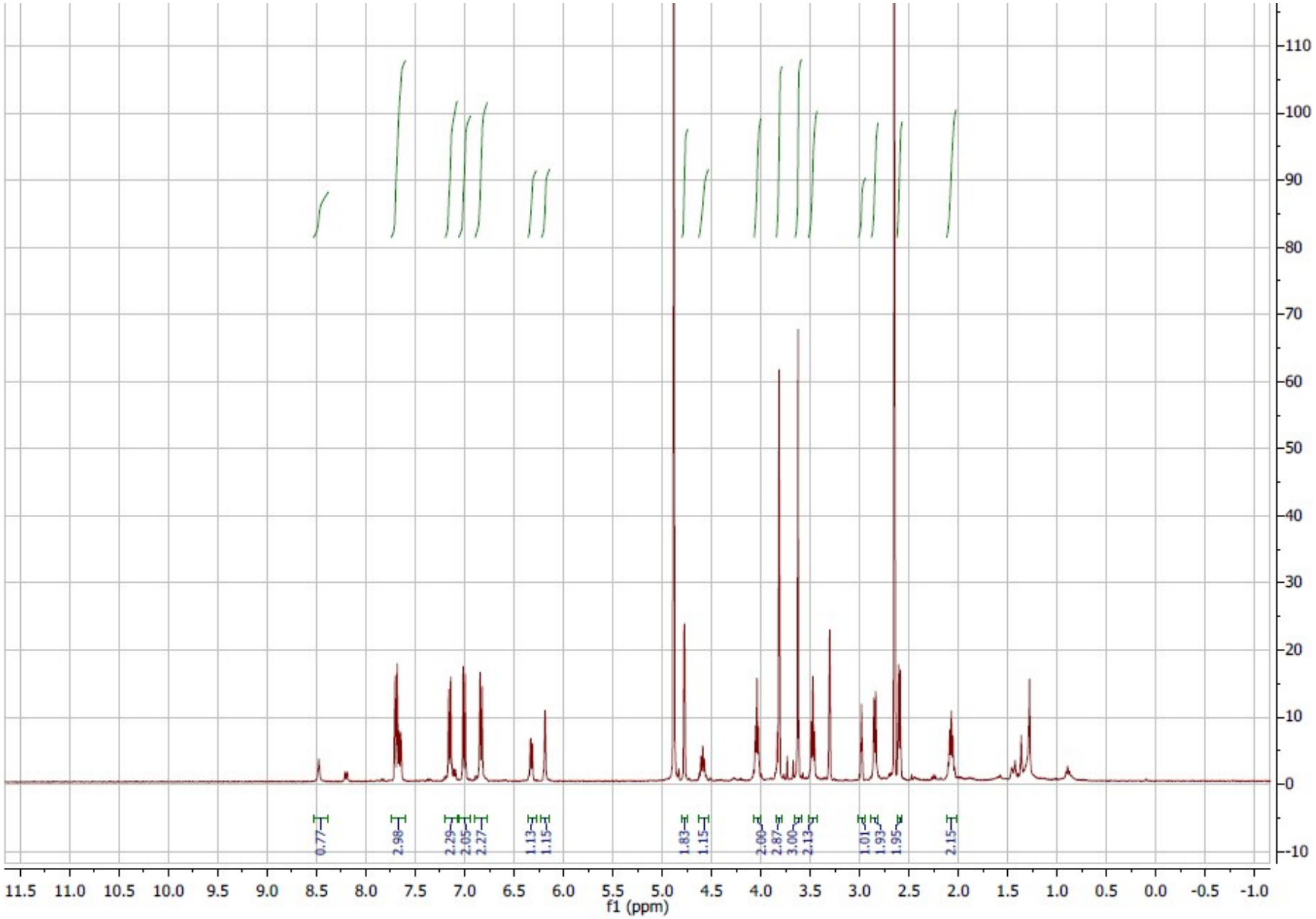


^{1}H NMR of **13** (400 MHz, CD_3OD)

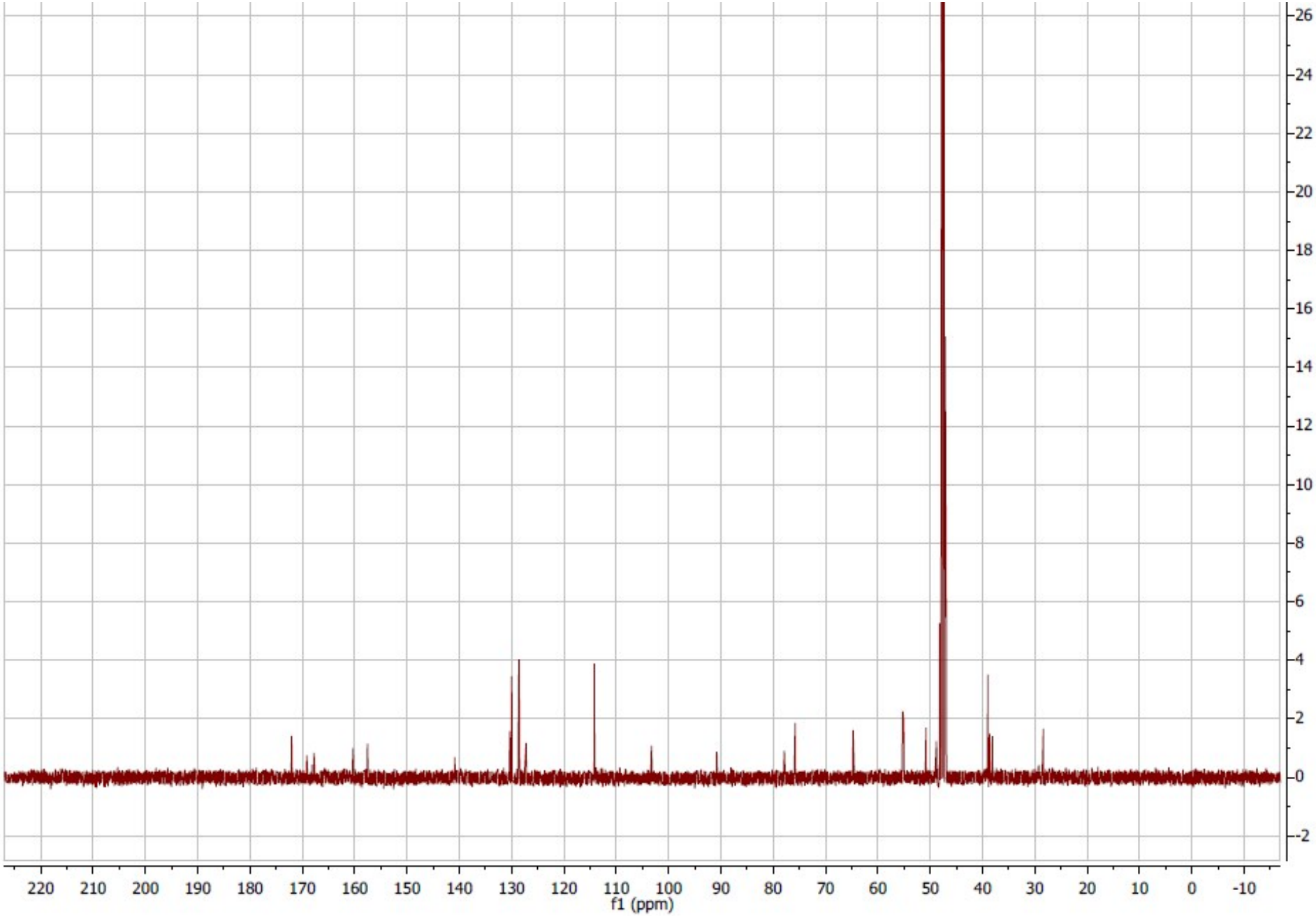




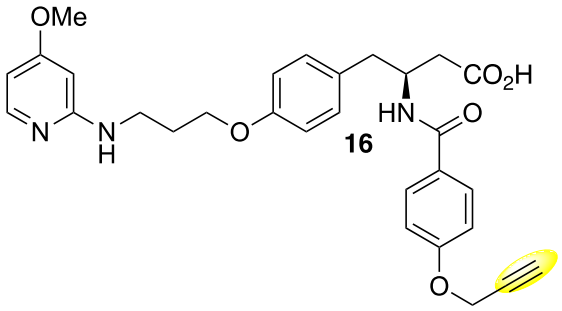
LC/MS of **15** (+ mode)

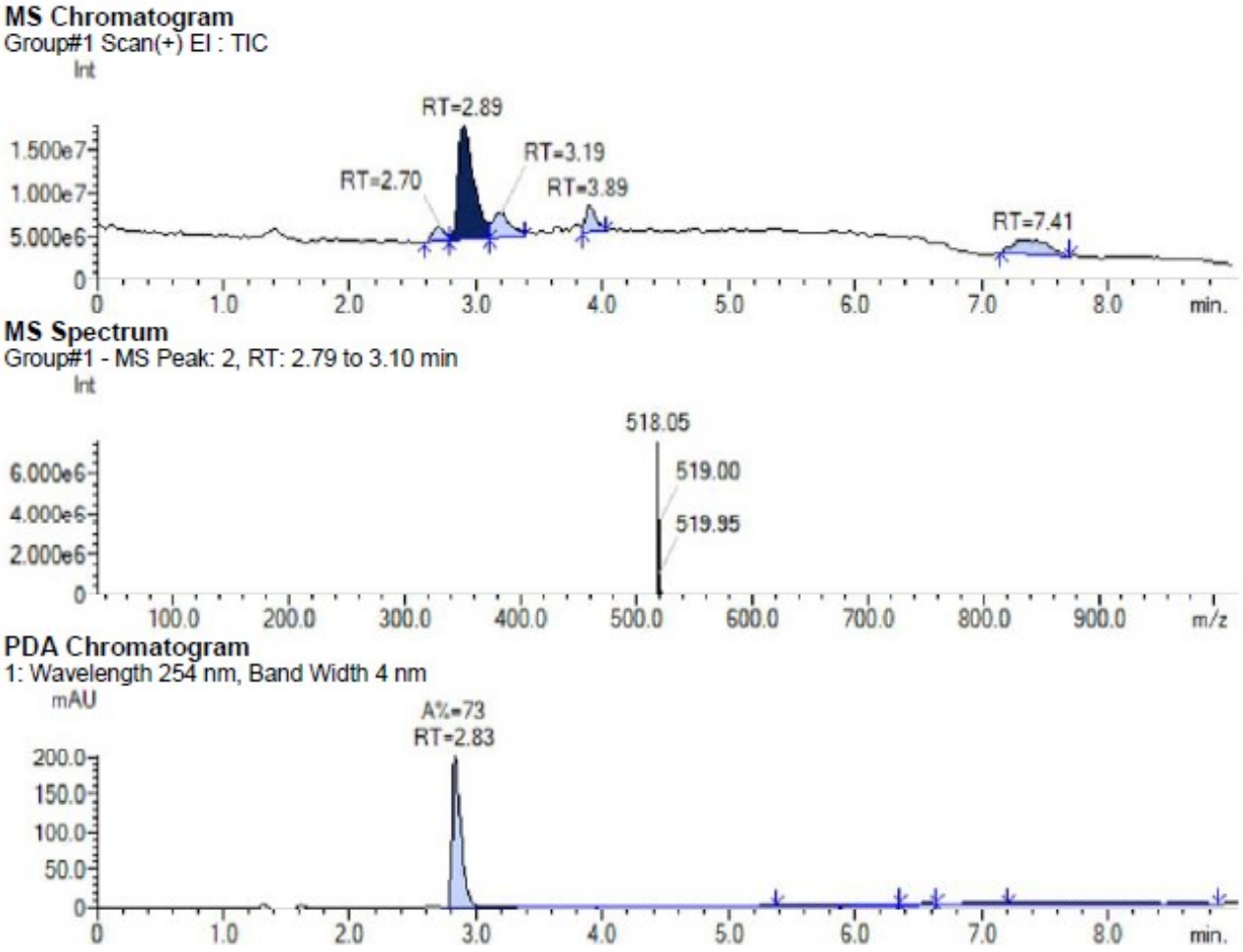

0.77
2.98
2.29
2.05
2.27
1.13
1.15
1.83
1.15
2.00
2.87
3.00
2.13
1.01
1.93
1.95
2.15
f1 (ppm)

^{1}H NMR of **15** (400 MHz, CD_3OD)

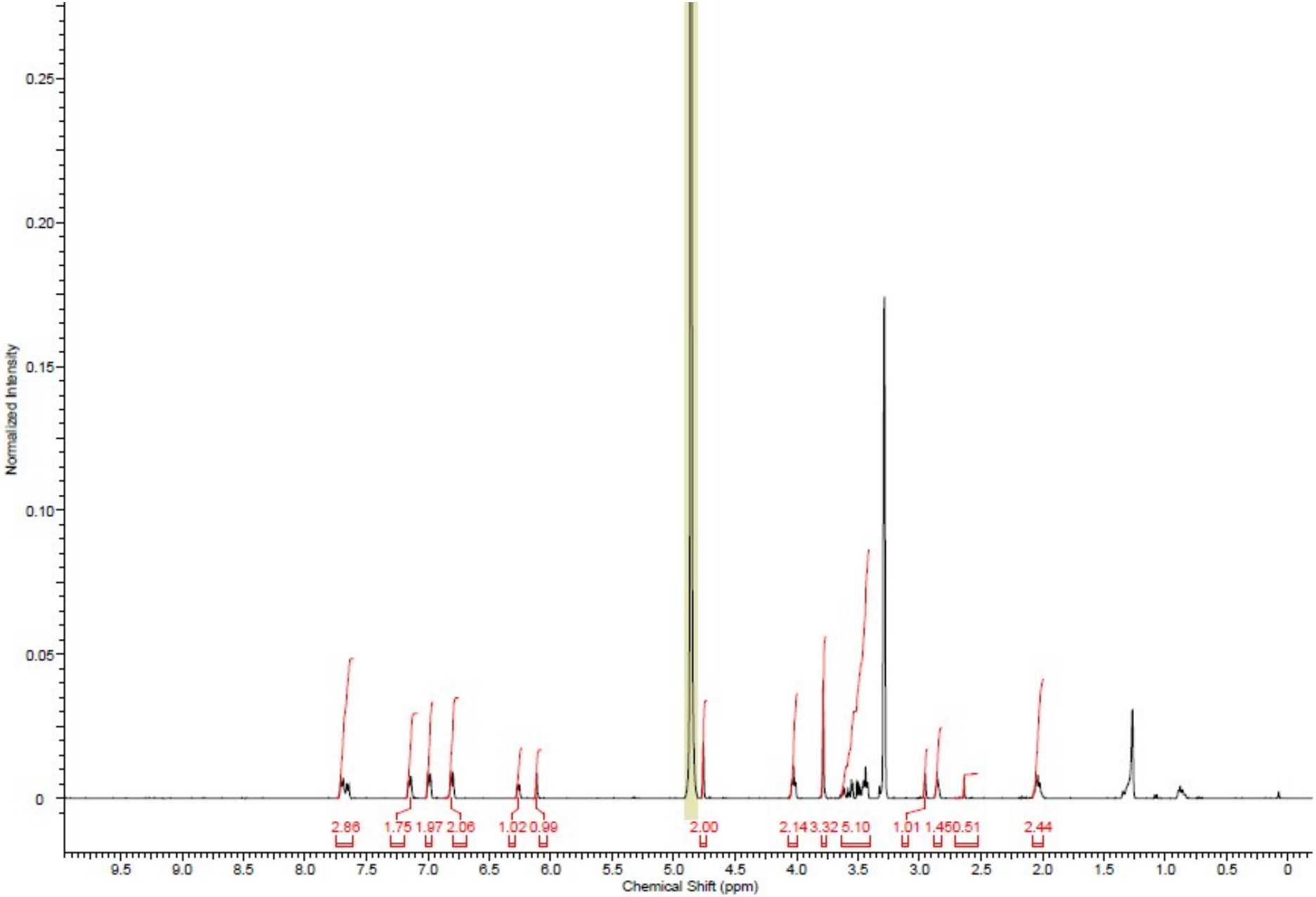

26
24
22
20
18
16
14
12
10
8
6
4
2
0
-2
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

^{13}C NMR of **15** (101 MHz, CD_3OD)

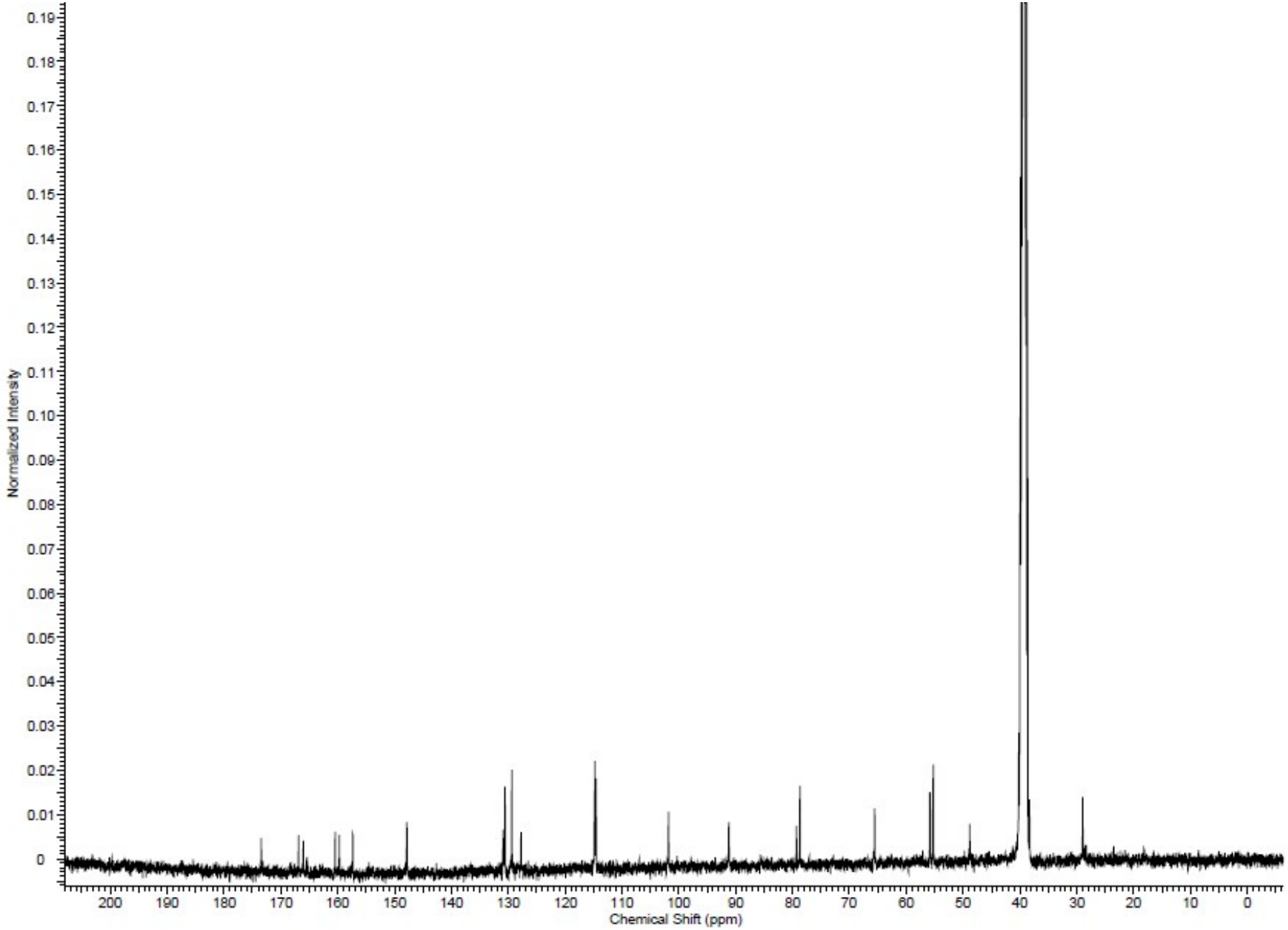




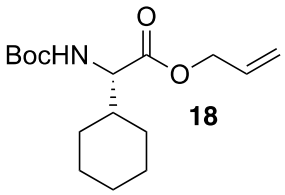
LC/MS of **16** (+ mode)

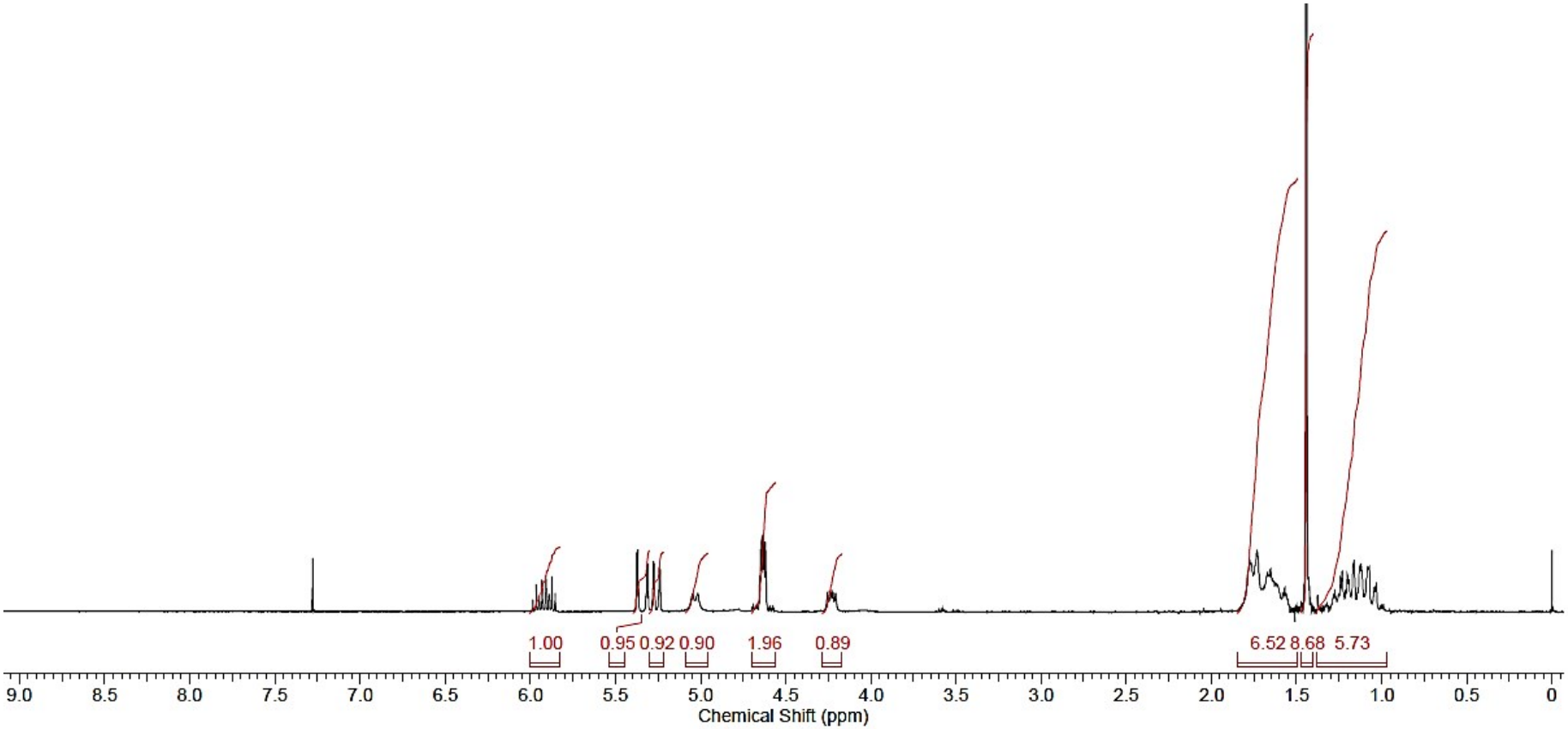


^{1}H NMR of **16** (400 MHz, CD_3OD)

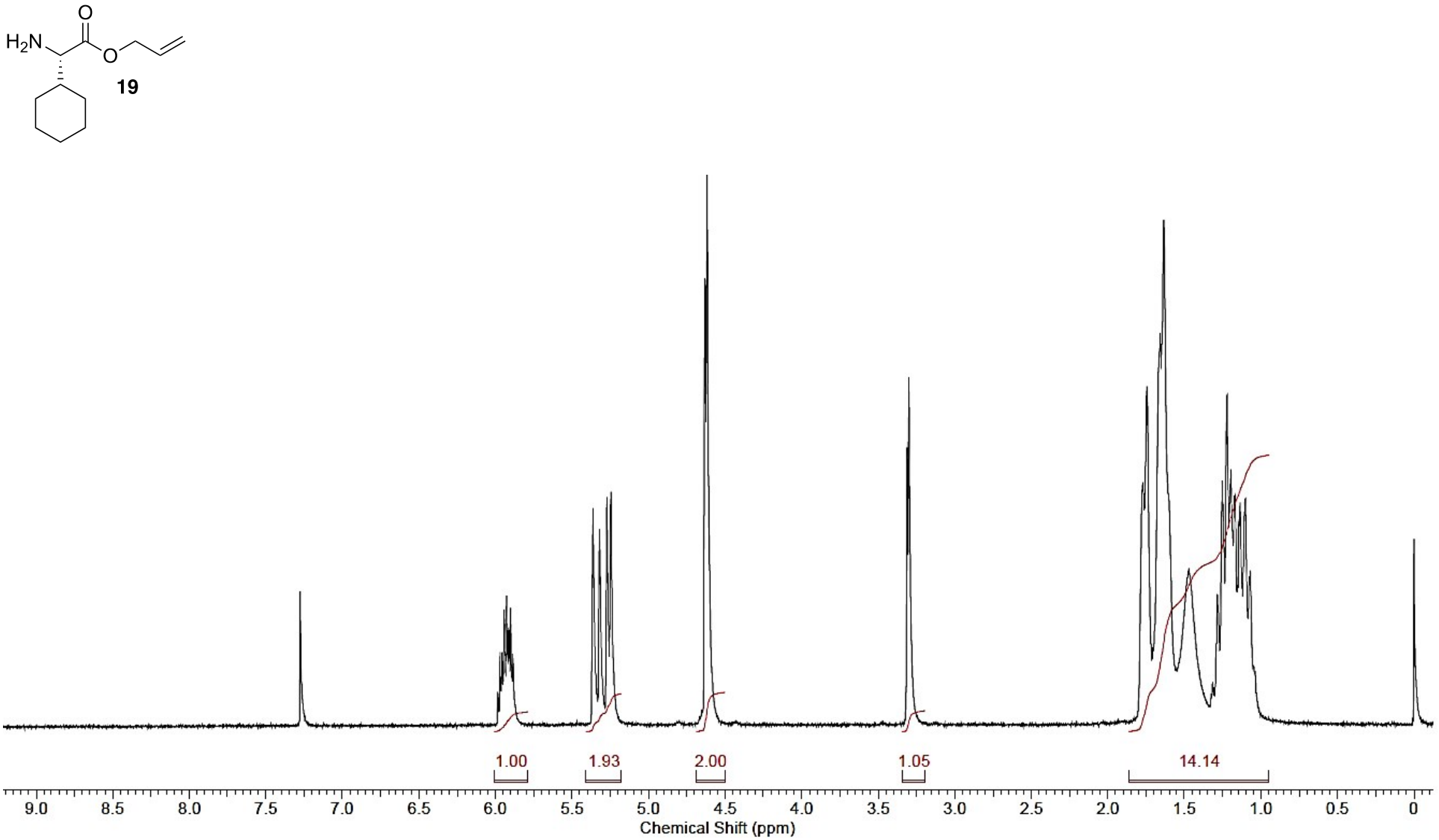


^{13}C NMR of **16** (101 MHz, DMSO-d_6)

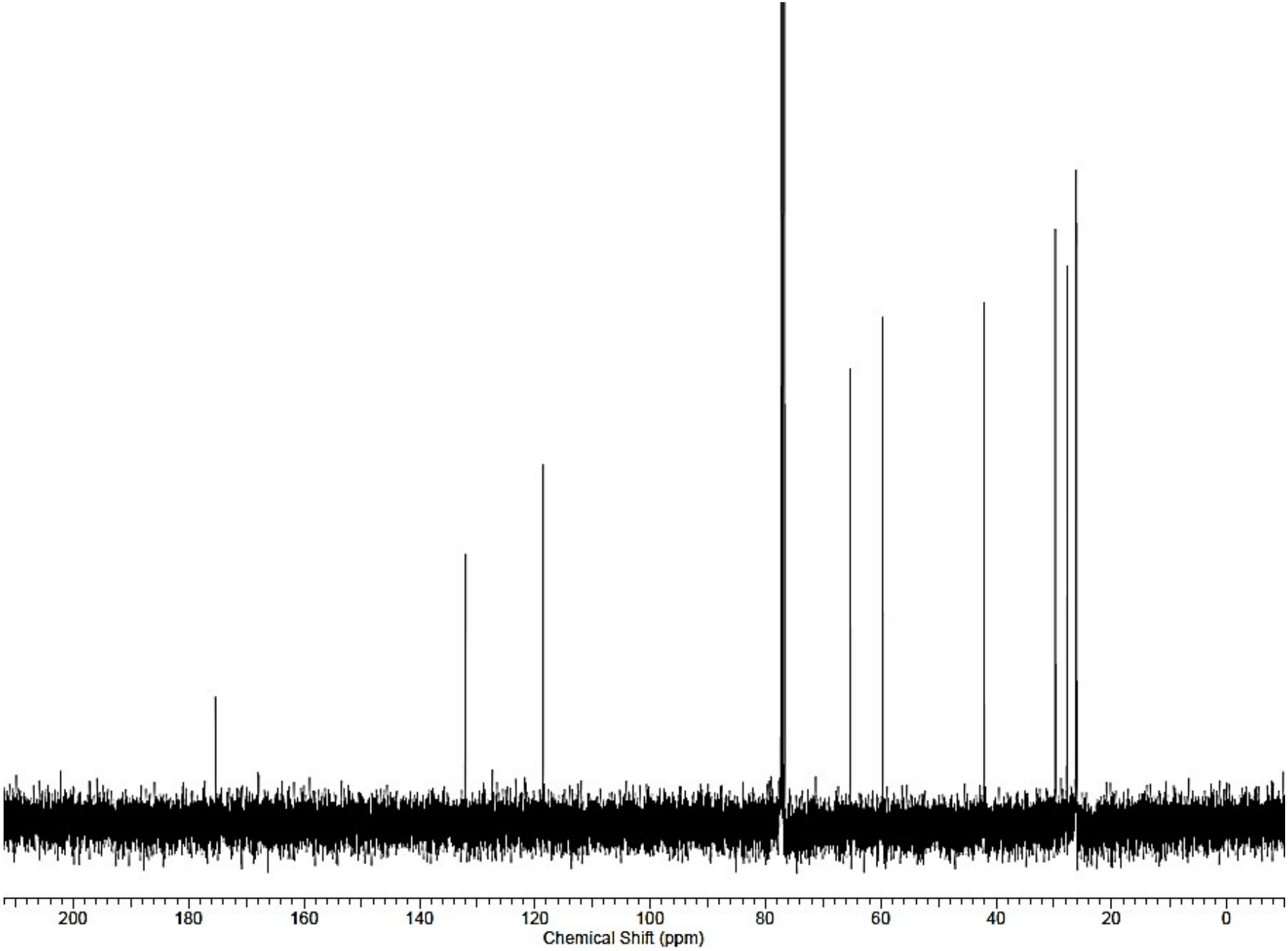




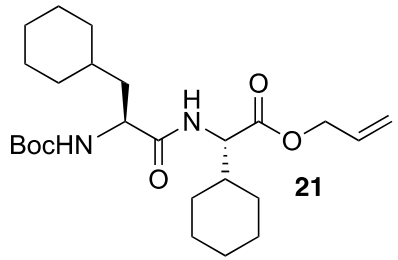
^{1}H NMR (300 MHz, $CDCl_3$) of **18**



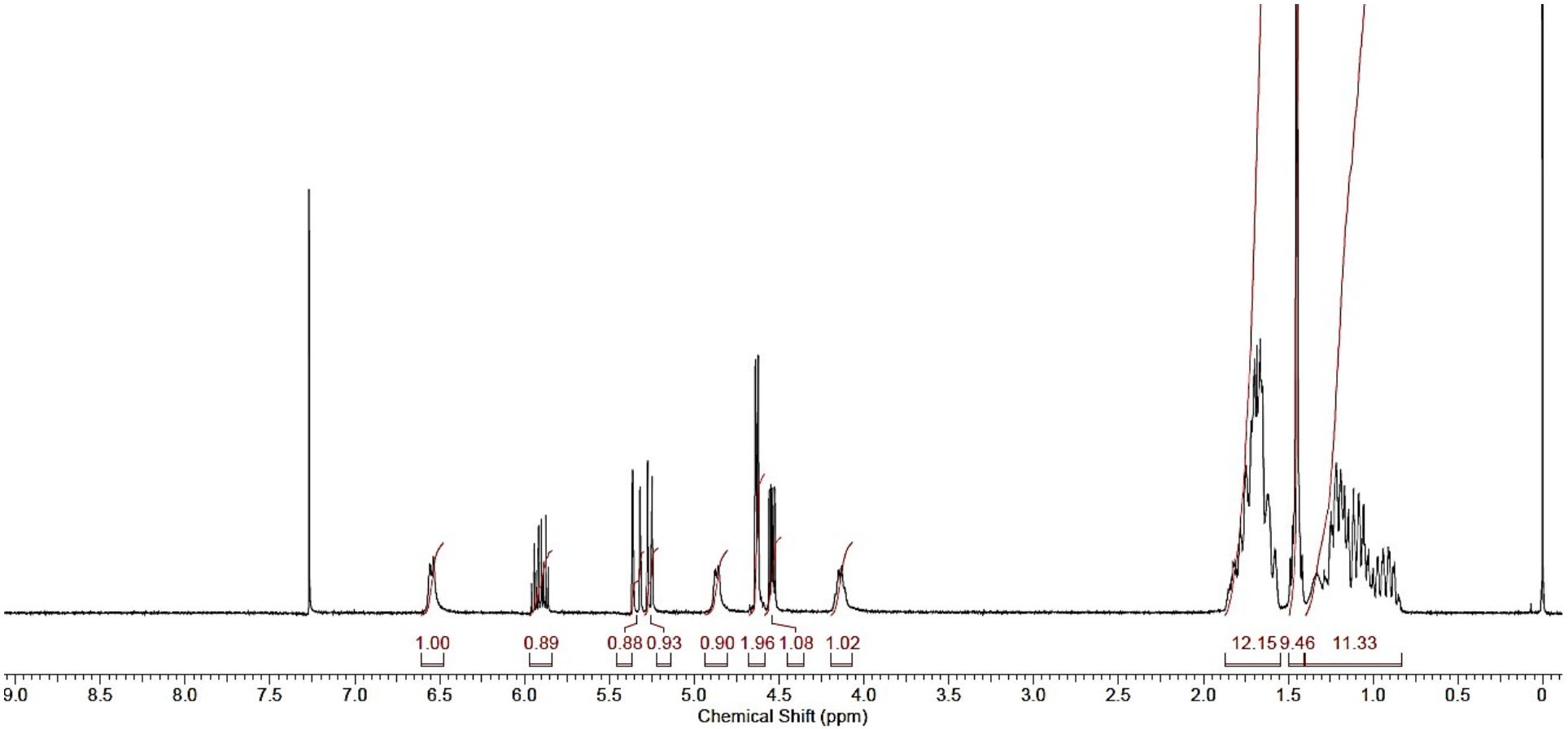
^{1}H NMR (400 MHz, $CDCl_3$) of **19**



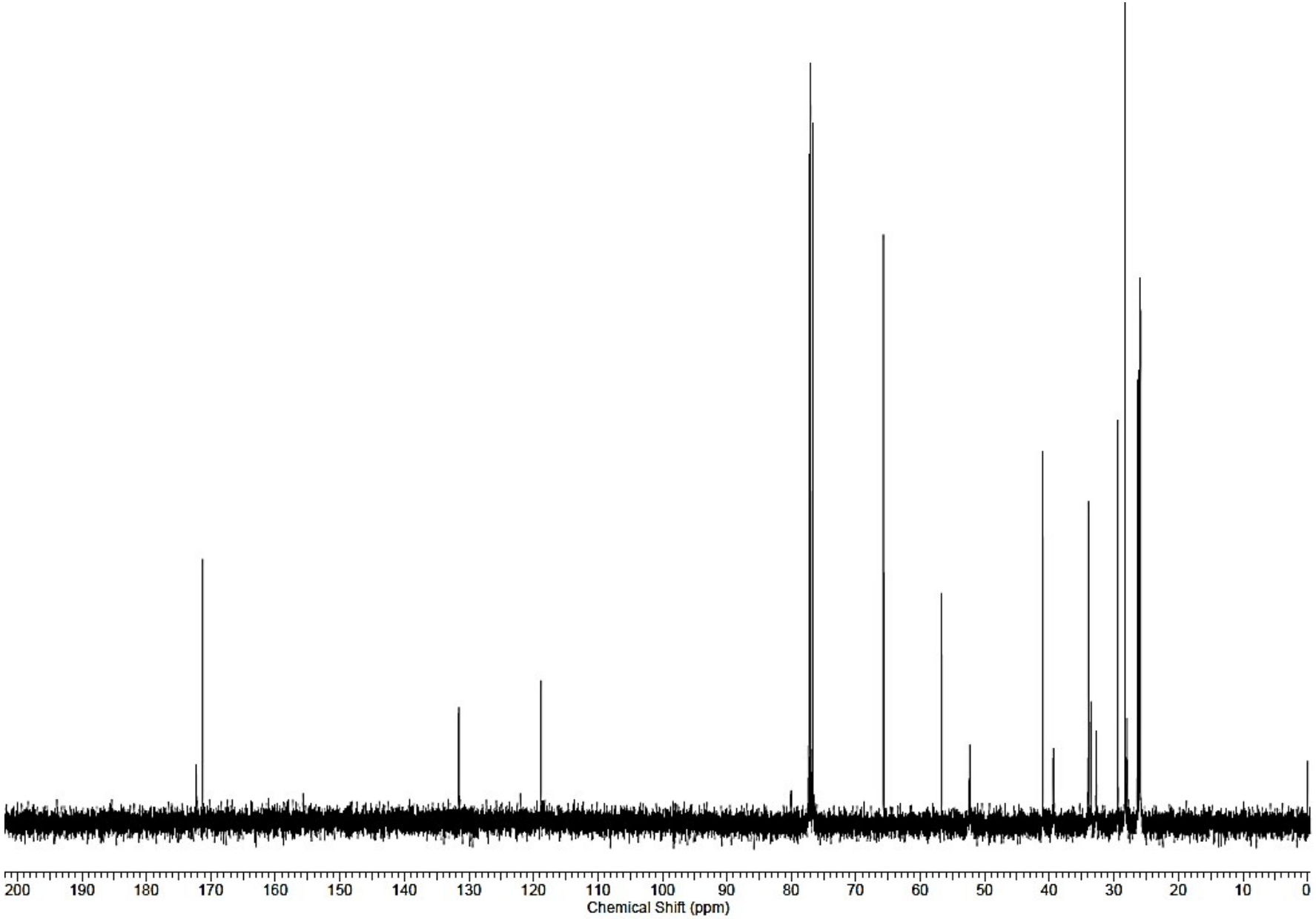
^{13}C NMR (125 MHz, $CDCl_3$) of **19**



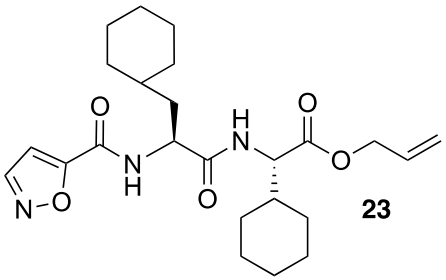
21



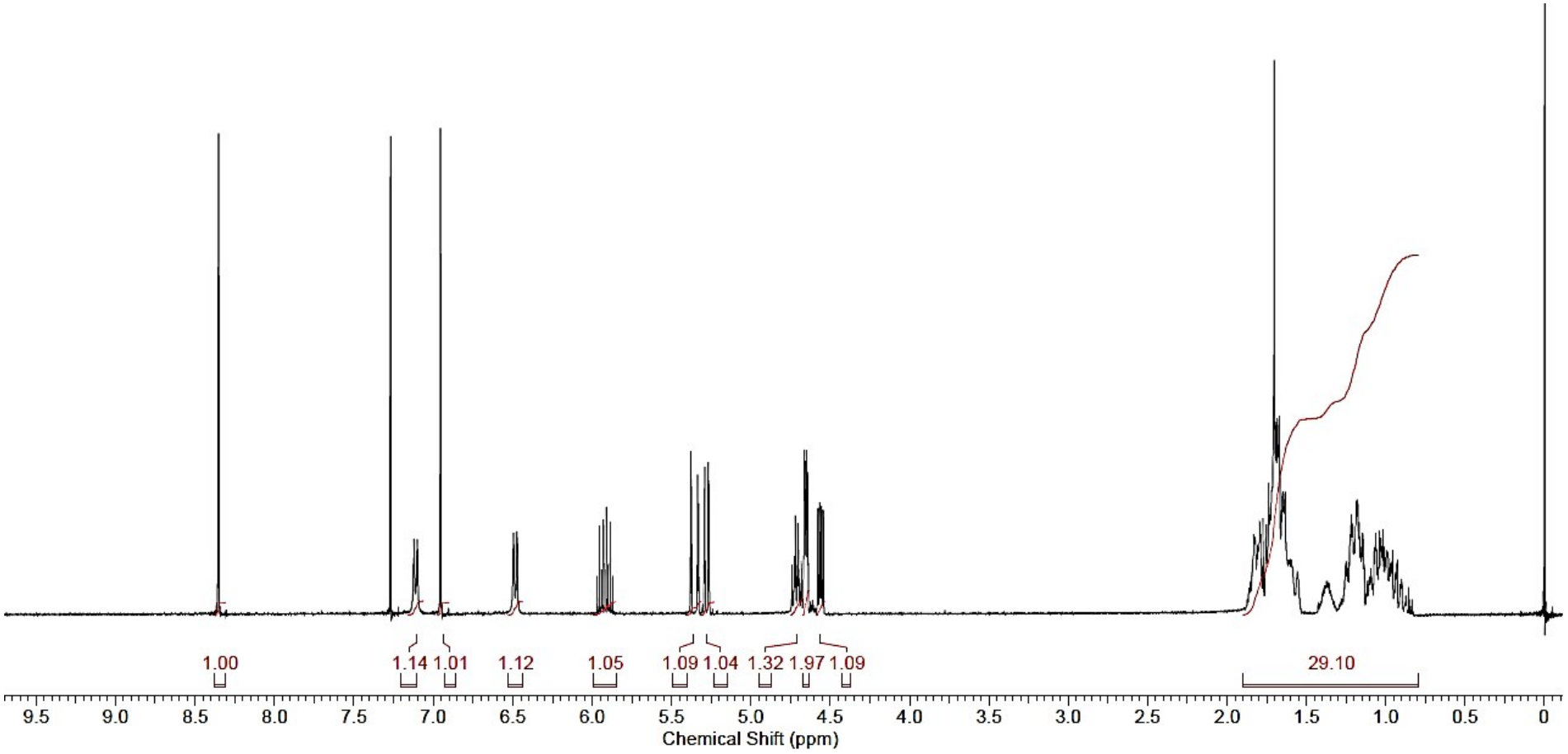
^{1}H NMR (400 MHz, $CDCl_3$) of **21**



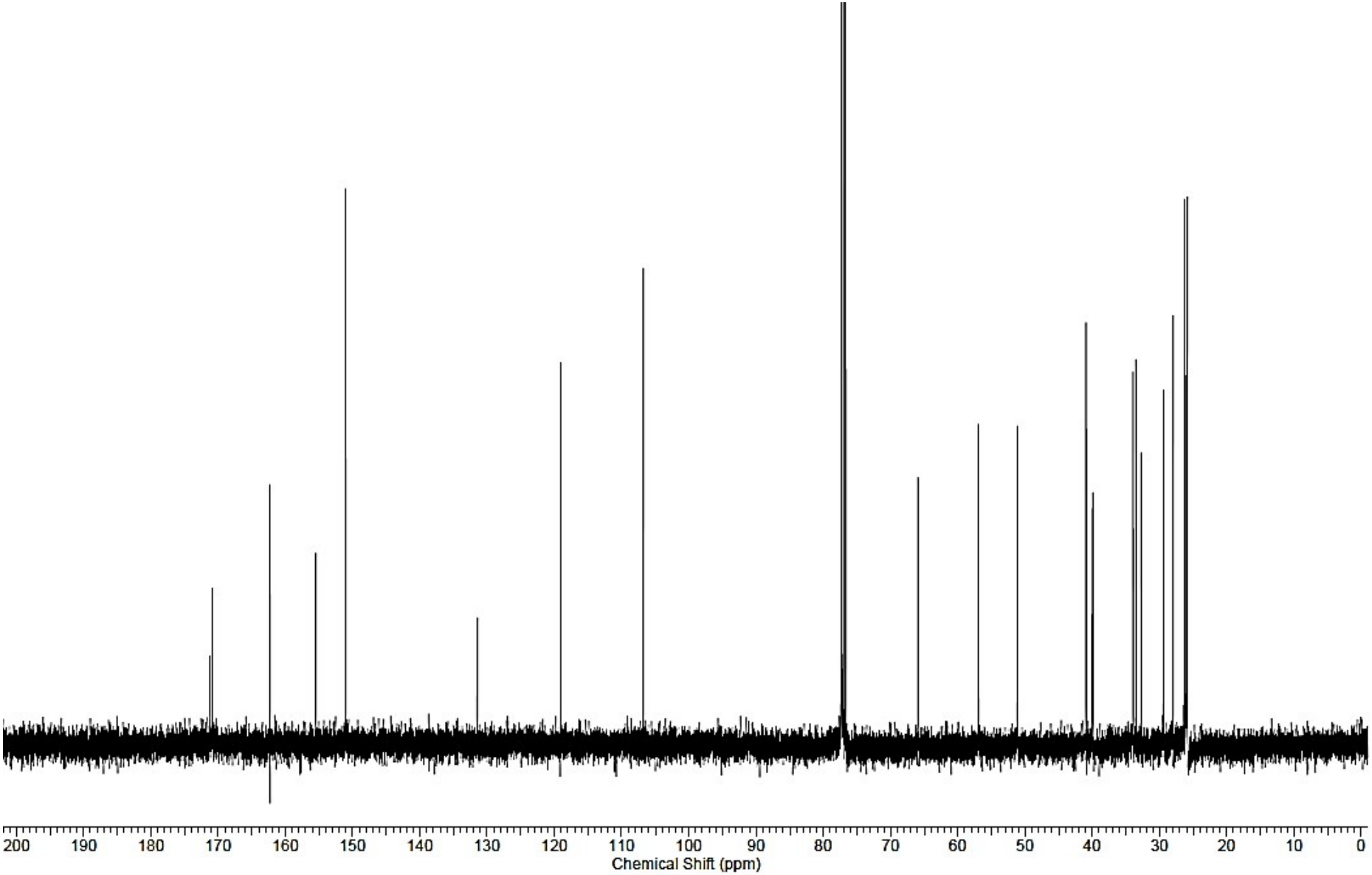
^{13}C NMR (125 MHz, $CDCl_3$) of **21**



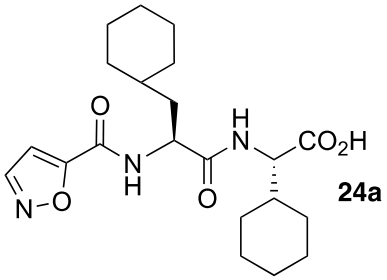
23



^{1}H NMR (400 MHz, $CDCl_3$) of **23**



^{13}C NMR (125 MHz, $CDCl_3$) of **23**

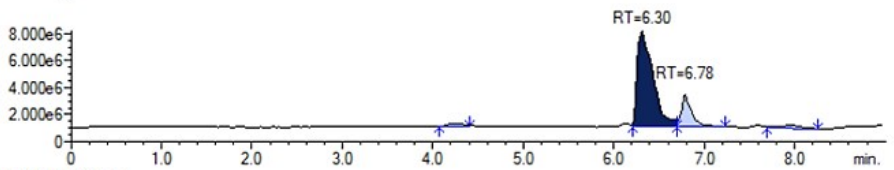


24a

Data File Name:	C:\Data\docken\Mark M\031516_MWM_PAR_027_A1.lcd
Sample Location:	Plate Number: 1 - Position: 26
Run By:	MarkM
Run Started:	Tuesday, March 15, 2016 3:59:33 PM
Run Finished:	Tuesday, March 15, 2016 4:09:35 PM
Method:	Standard Gemini

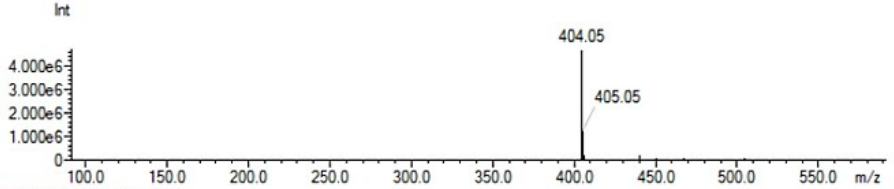
MS Chromatogram

Group#2 Scan(-) EI : TIC



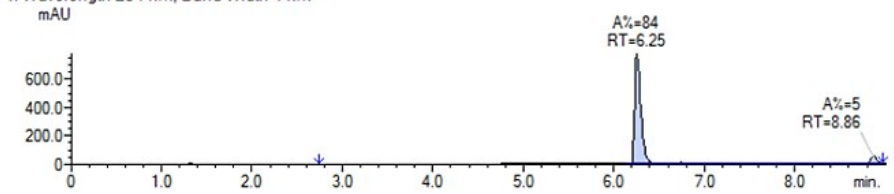
MS Spectrum

Group#2 - MS Peak: 7, RT: 6.20 to 6.69 min

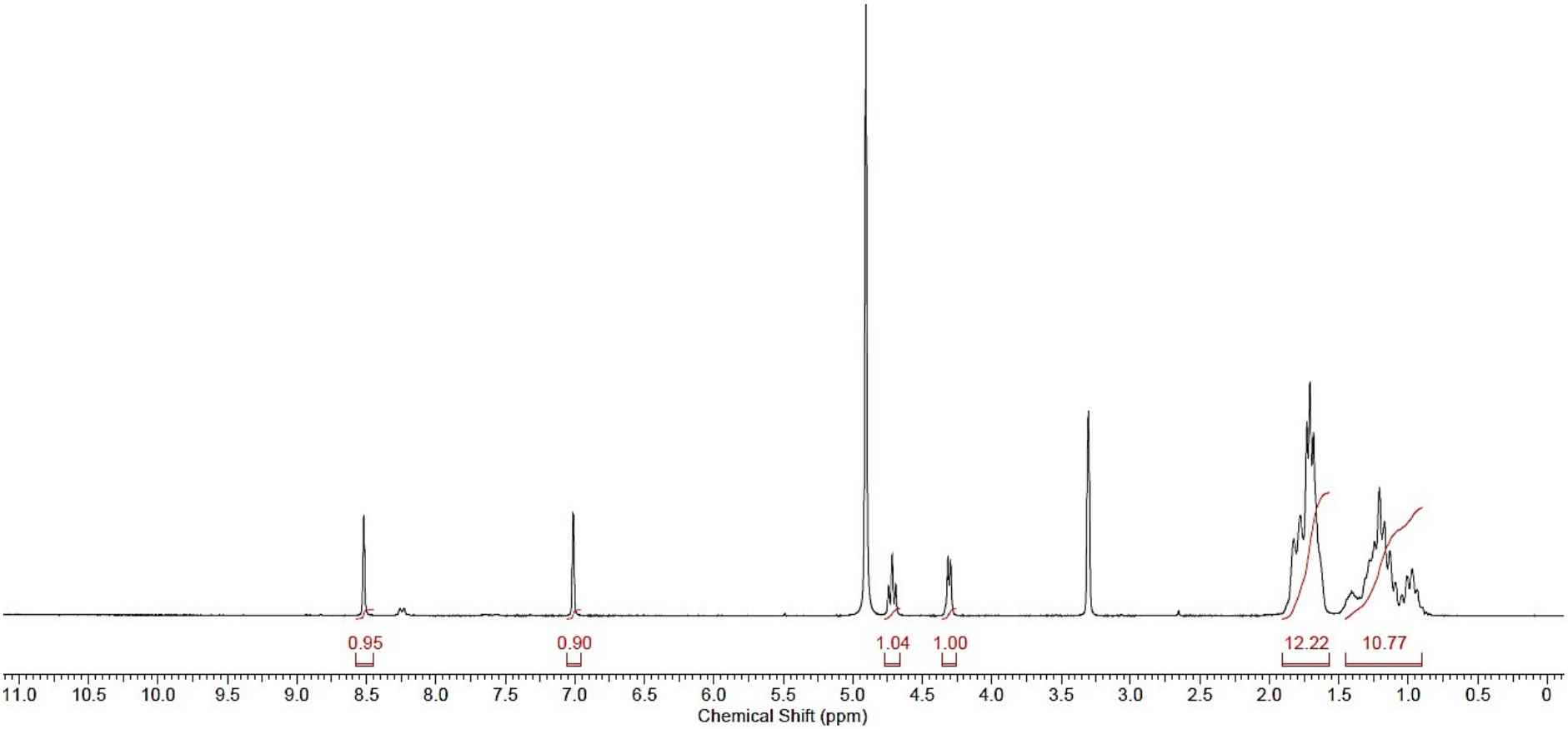


PDA Chromatogram

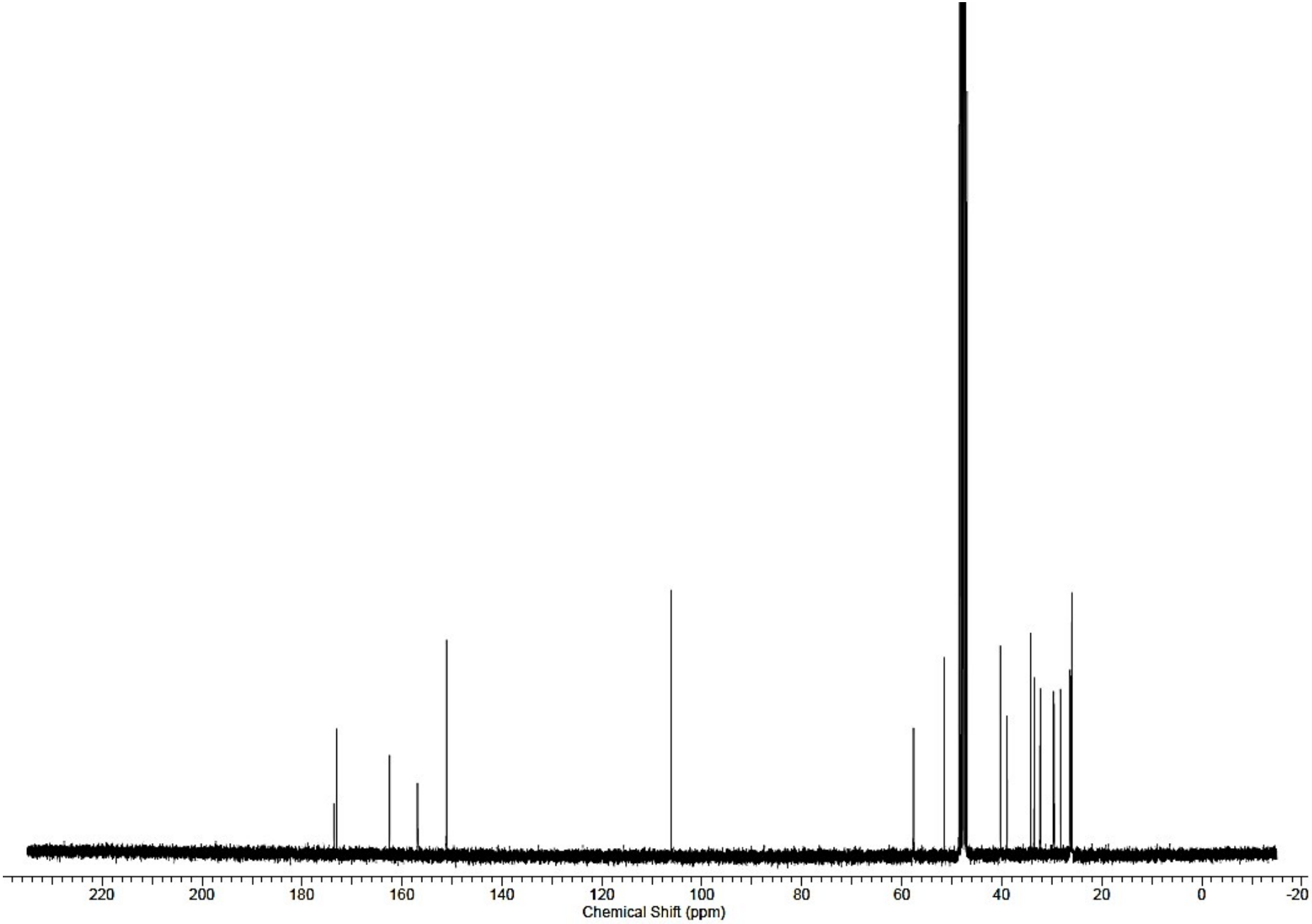
1: Wavelength 254 nm, Band Width 4 nm



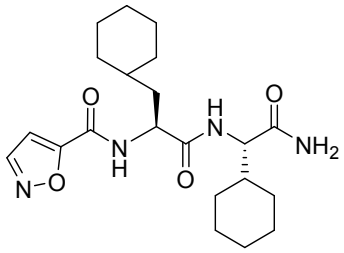
LC/MS of **24a** (- mode)



^{1}H NMR (300 MHz, CD_3OD) of **24a**



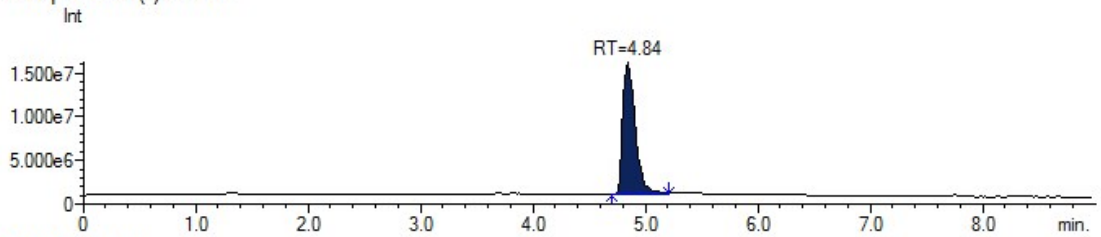
^{13}C NMR (75 MHz, CD_3OD) of **24a**



Sample:	072418_MWM_PAR_334_Diastereomer1
Sample Description:	072418_MWM_PAR_334_Diastereomer1
Data File Name:	C:\Data\docken\Mark M\072418_MWM_PAR_334_Diastereomer1.lcd
Sample Location:	Plate Number: 1 - Position: 104
Run By:	MarkM
Run Started:	Tuesday, July 24, 2018 4:01:36 PM
Run Finished:	Tuesday, July 24, 2018 4:14:42 PM
Method:	051817_Std_Gemini_25_MeCN

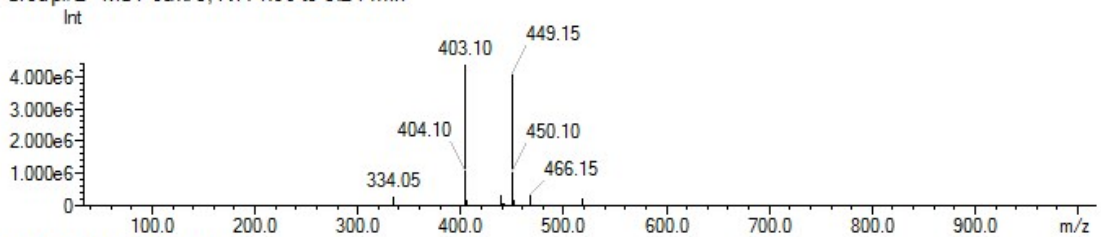
MS Chromatogram

Group#2 Scan(-) EI : TIC



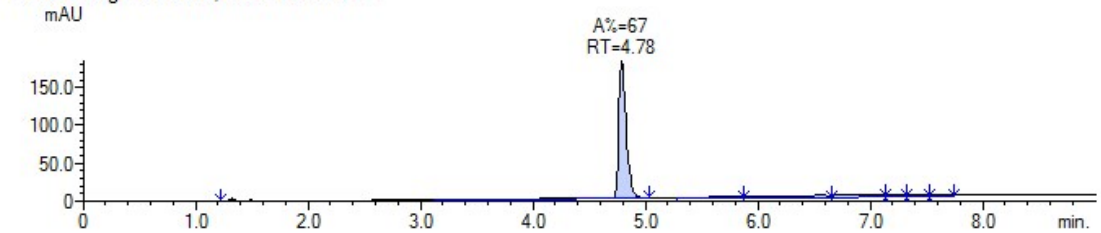
MS Spectrum

Group#2 - MS Peak: 5, RT: 4.69 to 5.21 min

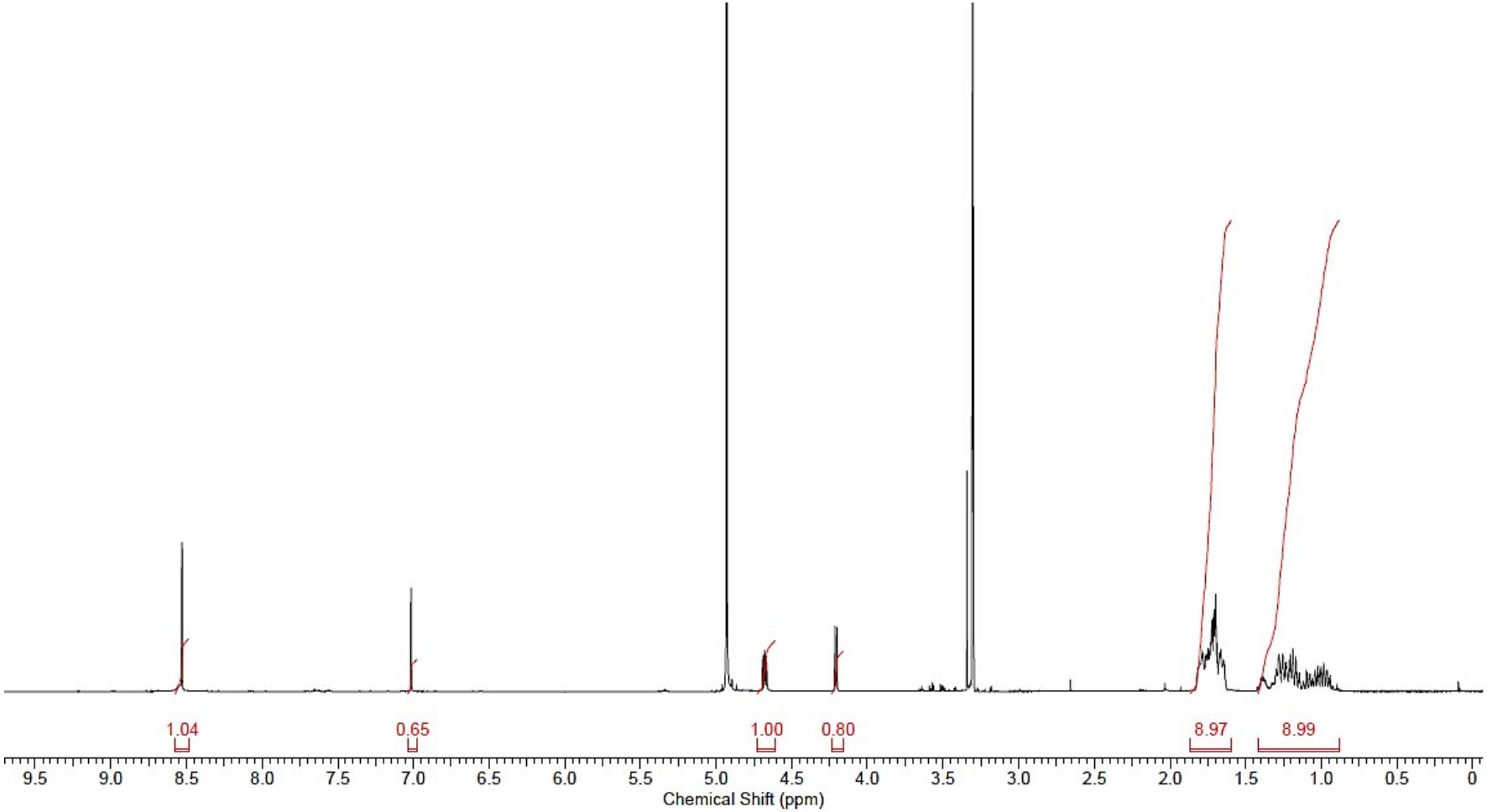


PDA Chromatogram

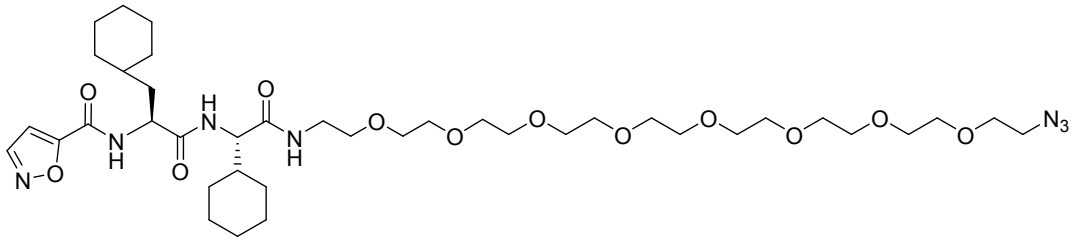
1: Wavelength 254 nm, Band Width 4 nm



LC/MS of **24b** (- mode)



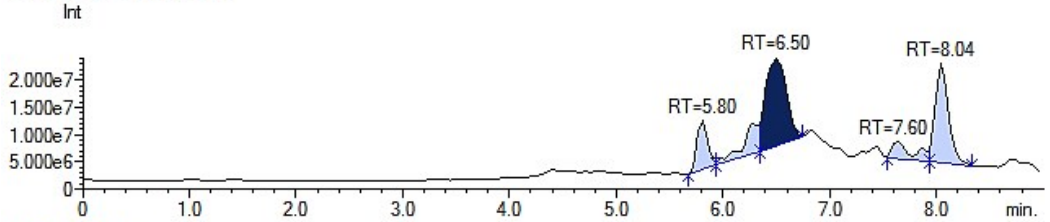
^{1}H NMR of **24b** (600 MHz. CD_3OD)



Sample:	051016_MWM_PAR_063_Prep_Check_2
Sample Description:	051016_MWM_PAR_063_Prep_Check_2
Data File Name:	C:\Data\docken\Mark M\051016_MWM_PAR_063_Prep_Check_2.lcd
Sample Location:	Plate Number: 1 - Position: 63
Run By:	MarkM
Run Started:	Tuesday, May 10, 2016 5:44:36 PM
Run Finished:	Tuesday, May 10, 2016 5:54:26 PM
Method:	Standard Gemini - High Mass 9 min MeOH

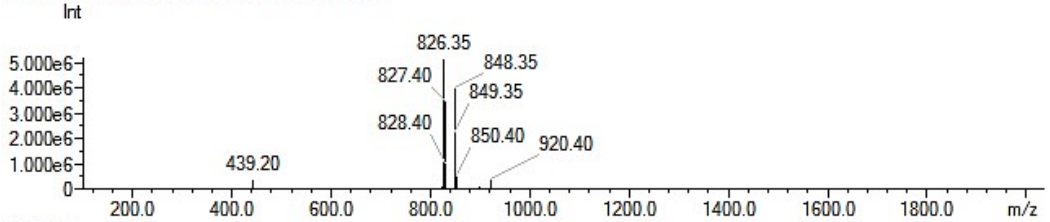
MS Chromatogram

Group#2 Scan(+) EI : TIC



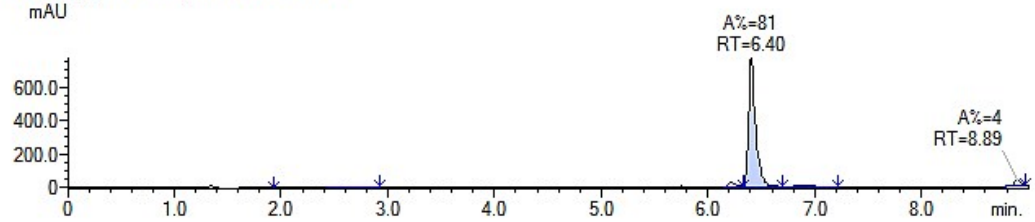
MS Spectrum

Group#2 - MS Peak: 8, RT: 6.33 to 6.73 min

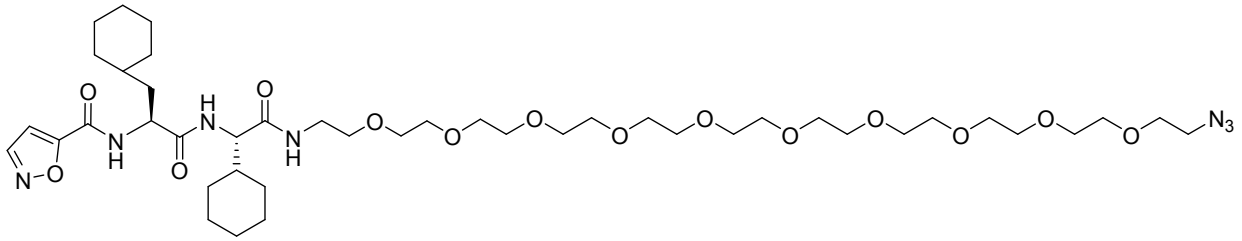


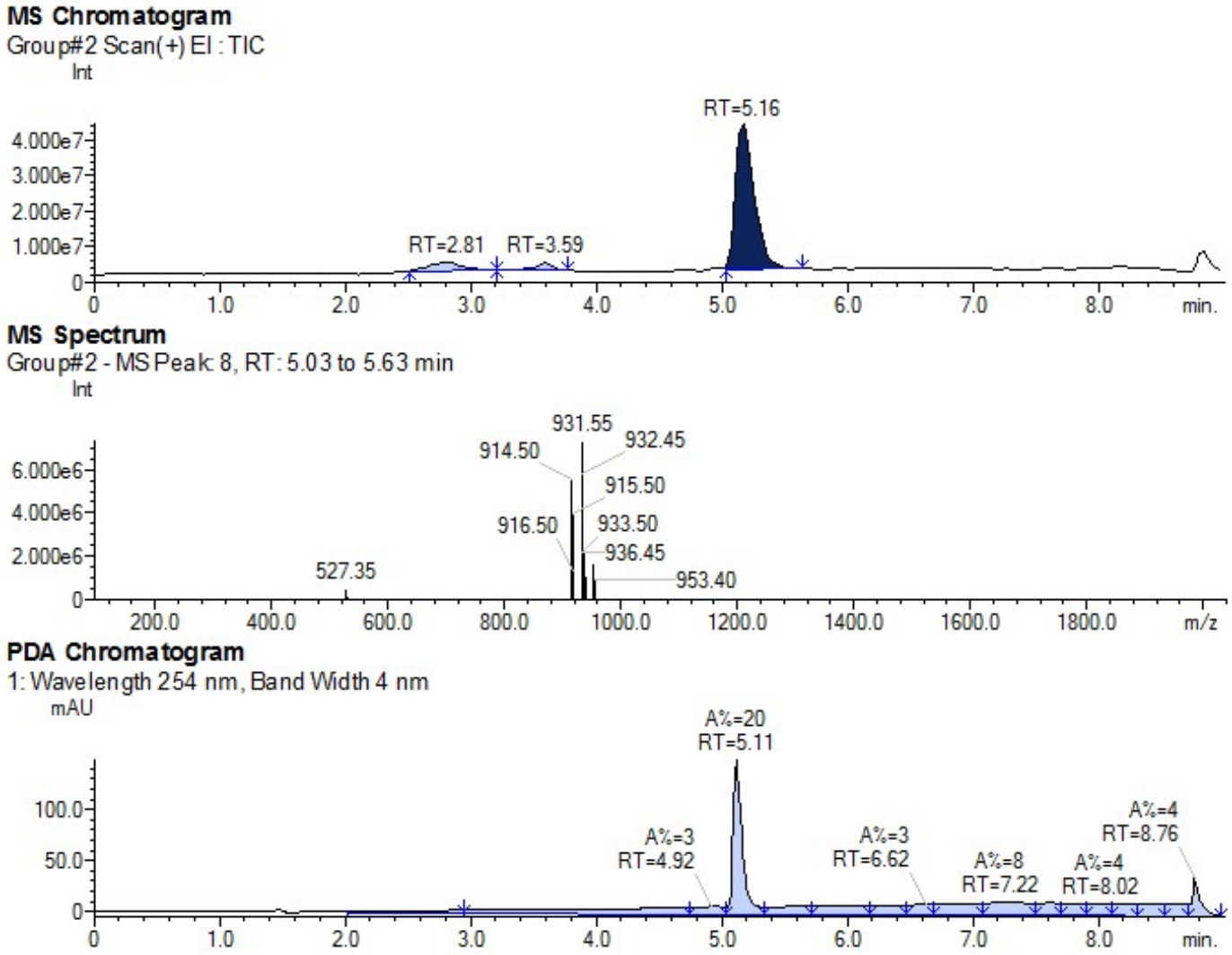
PDA Chromatogram

1: Wavelength 254 nm, Band Width 4 nm

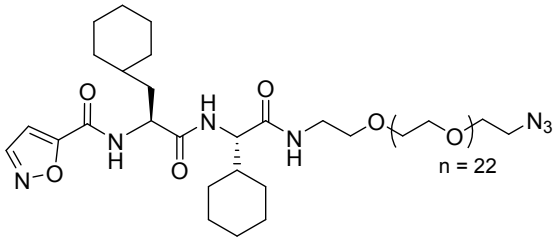


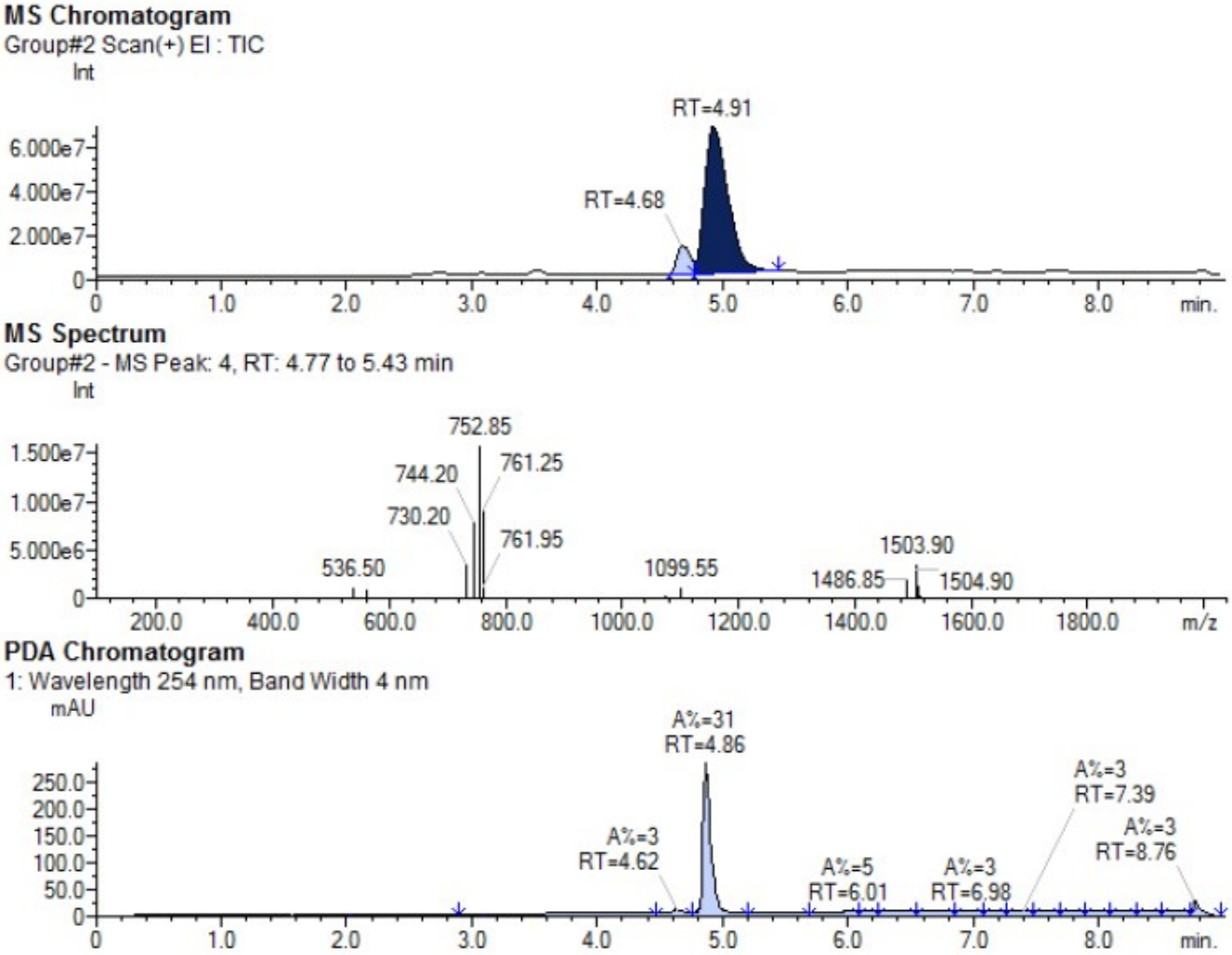
LC/MS of **29b** (+ mode)



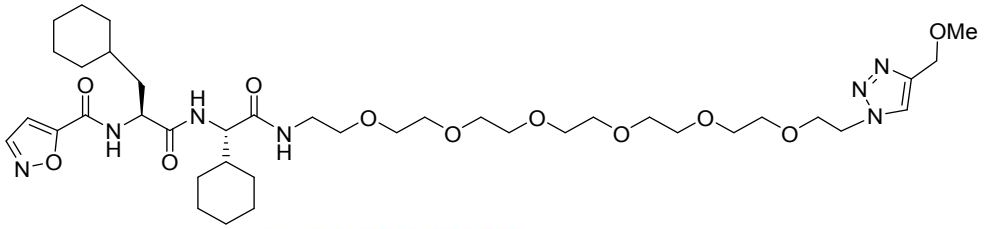


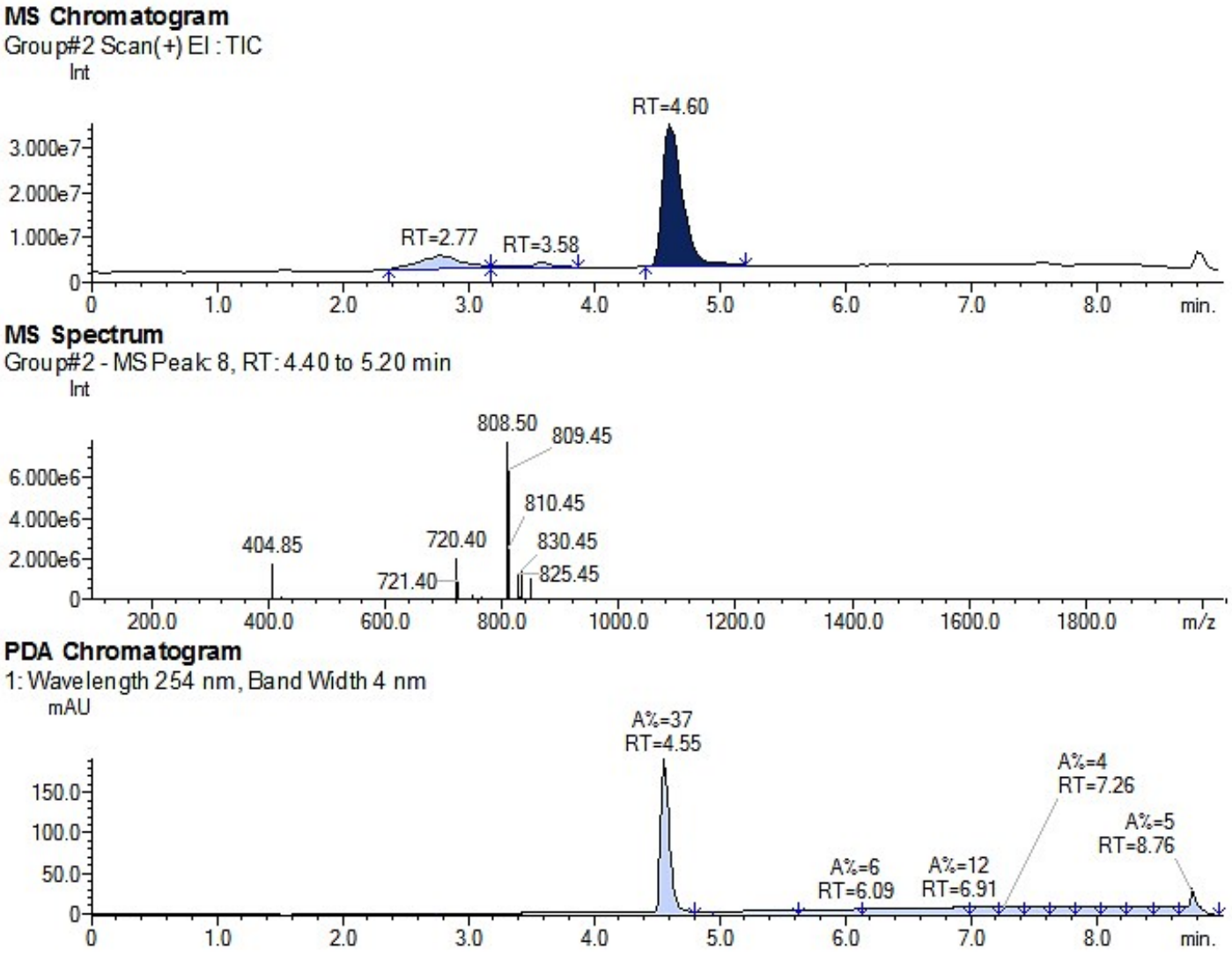
LC/MS of **29c** (+ mode)



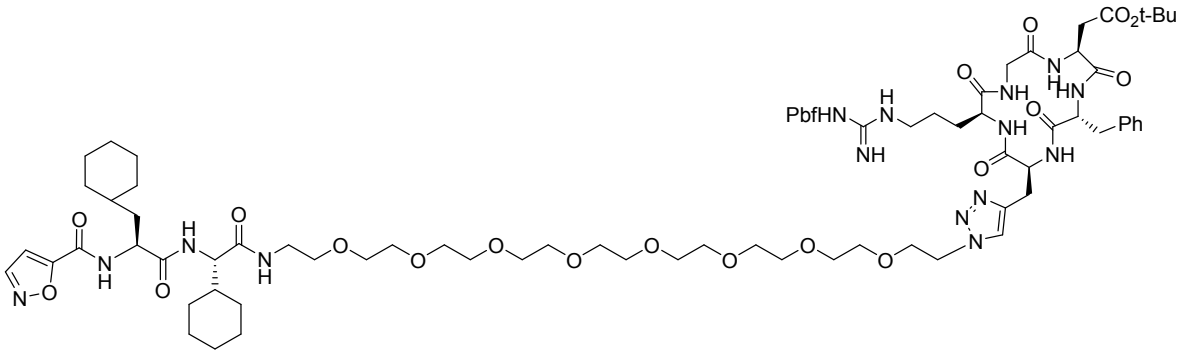


LC/MS of **29d** (+ mode)





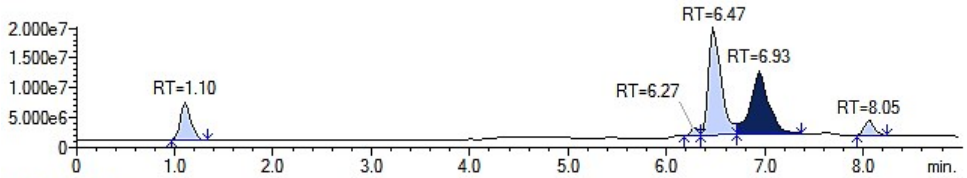
LC/MS of **30** (+ mode)



Data File Name:	C:\Data\docken\Mark M\051016_MWM_PAR_067_Prep_Check.lcd
Sample Location:	Plate Number: 1 - Position: 67
Run By:	MarkM
Run Started:	Tuesday, May 10, 2016 5:34:32 PM
Run Finished:	Tuesday, May 10, 2016 5:44:34 PM
Method:	Standard Gemini - High Mass 9 min MeOH

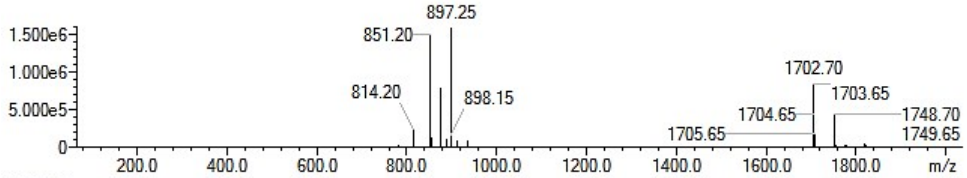
MS Chromatogram

Group#1 Scan(-) EI : TIC



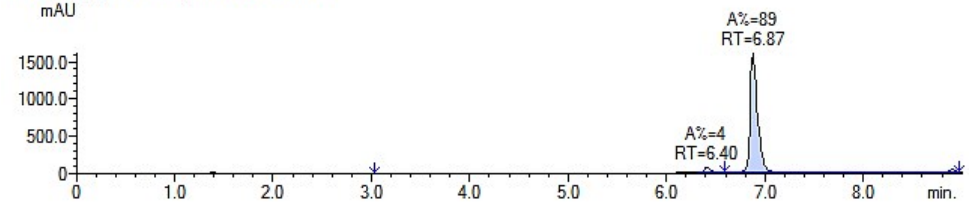
MS Spectrum

Group#1 - MS Peak: 4, RT: 6.70 to 7.37 min

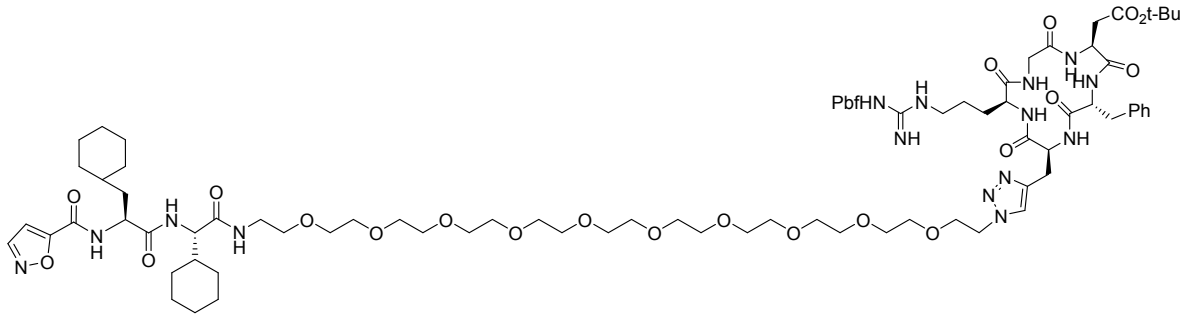


PDA Chromatogram

1: Wavelength 254 nm, Band Width 4 nm



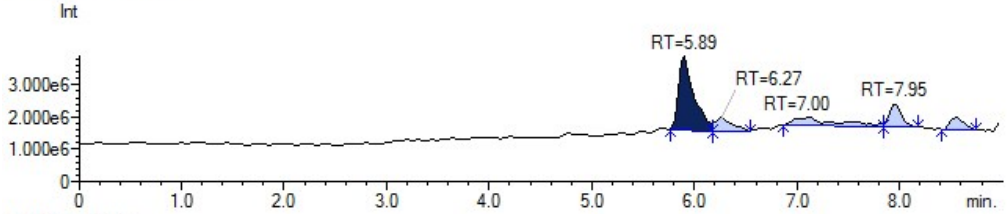
LC/MS of **31b** (- mode)



Sample:	072417_MWM_PAR_36_Check
Sample Description:	072417_MWM_PAR_36_Check
Data File Name:	C:\Data\docken\Mark M\072417_MWM_PAR_36_Check.lcd
Sample Location:	Plate Number: 1 - Position: 104
Run By:	MarkM
Run Started:	Monday, July 24, 2017 6:16:08 PM
Run Finished:	Monday, July 24, 2017 6:30:21 PM
Method:	Standard Gemini - High Mass 9 min MeOH

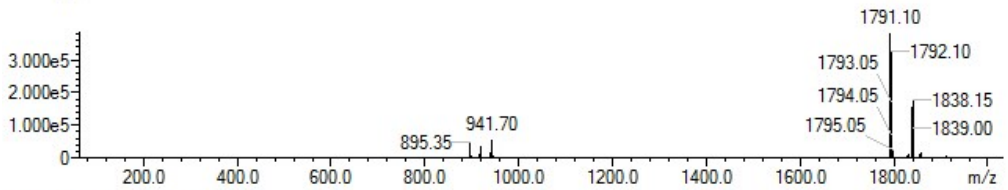
MS Chromatogram

Group#1 Scan(-) EI : TIC



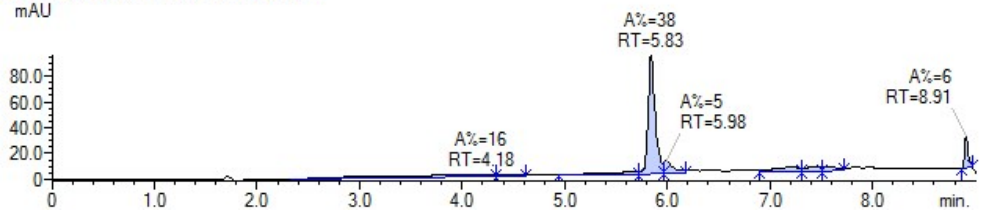
MS Spectrum

Group#1 - MS Peak: 1, RT: 5.77 to 6.17 min

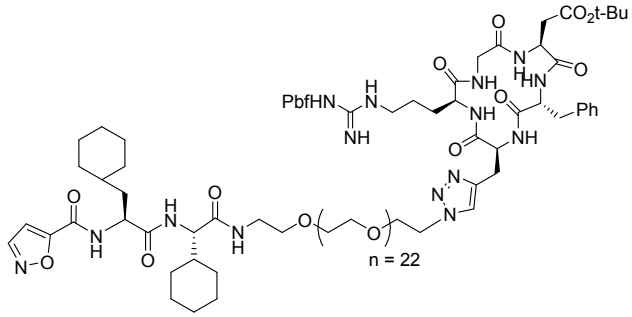


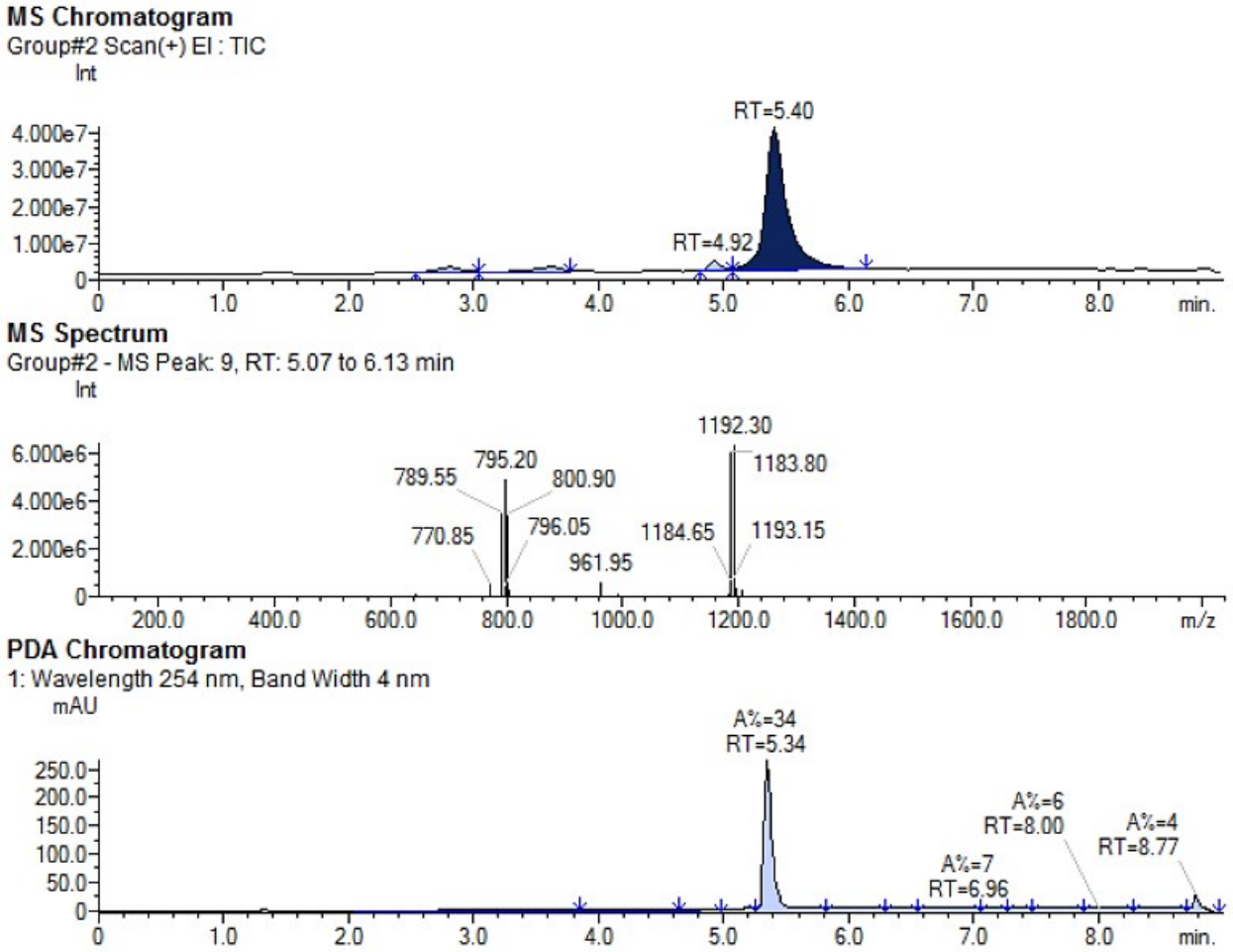
PDA Chromatogram

1: Wavelength 254 nm, Band Width 4 nm

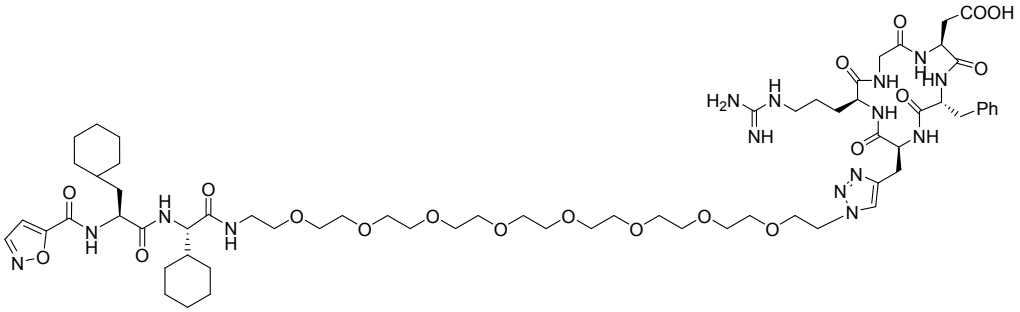


LC/MS of **31c** (- mode)





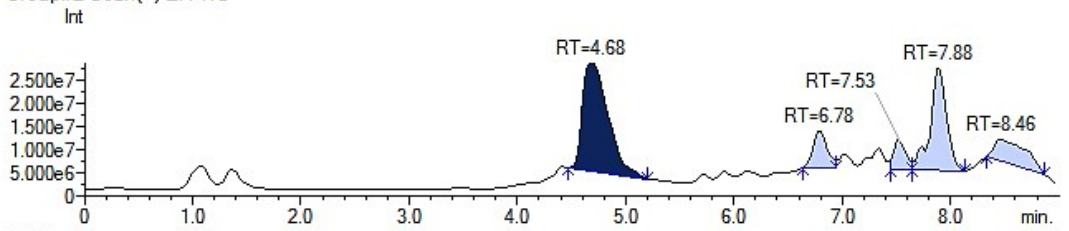
LC/MS of **31d** (+ mode)



Sample Location:	Plate Number: 1 - Position: 72
Run By:	MarkM
Run Started:	Monday, May 16, 2016 9:05:22 PM
Run Finished:	Monday, May 16, 2016 9:15:25 PM
Method:	Standard Gemini - High Mass 9 min MeOH

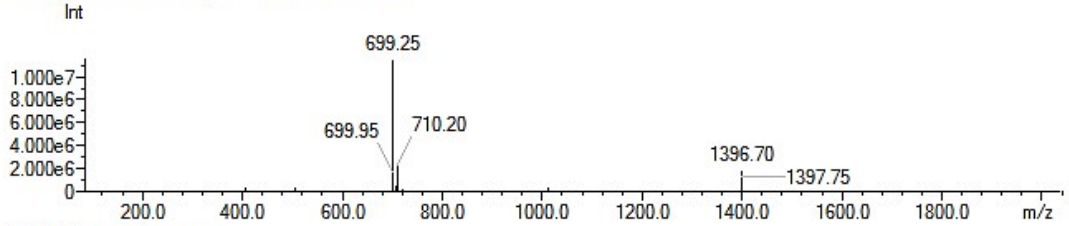
MS Chromatogram

Group#2 Scan(+) EI : TIC



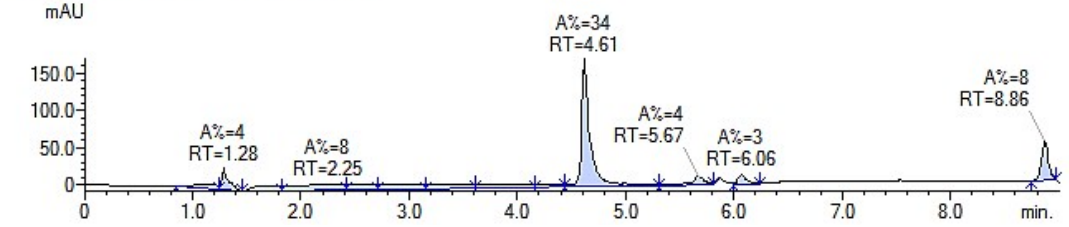
MS Spectrum

Group#2 - MS Peak: 6, RT: 4.47 to 5.20 min

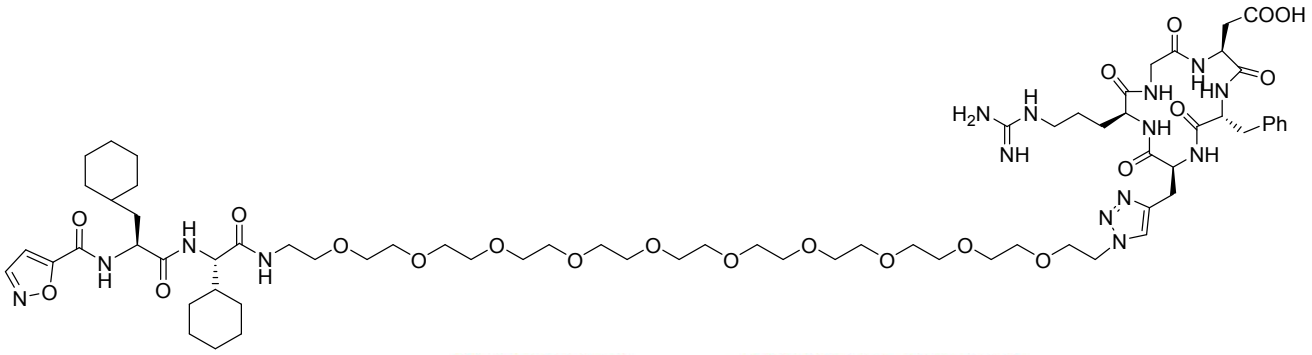


PDA Chromatogram

1: Wavelength 254 nm, Band Width 4 nm



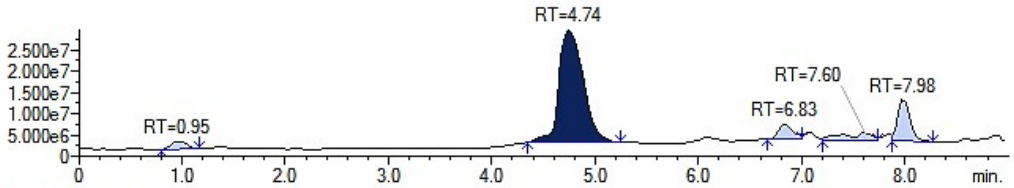
LC/MS of **32b** (+ mode)



Sample Location:	Plate Number: 1 - Position: 56
Run By:	MarkM
Run Started:	Tuesday, May 17, 2016 1:43:02 PM
Run Finished:	Tuesday, May 17, 2016 1:53:07 PM
Method:	Standard Gemini - High Mass 9 min MeOH

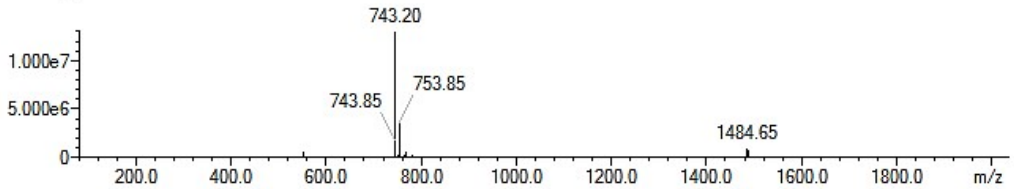
MS Chromatogram

Group#2 Scan(+) EI : TIC



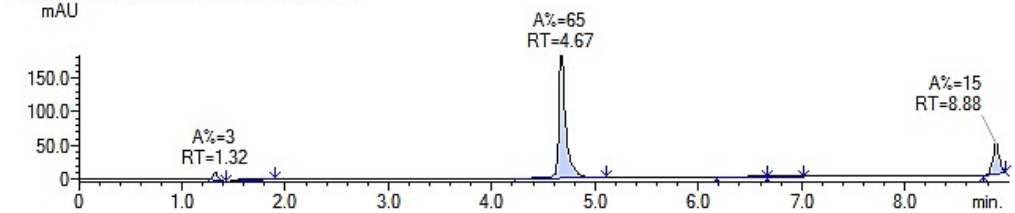
MS Spectrum

Group#2 - MS Peak: 7, RT: 4.33 to 5.23 min

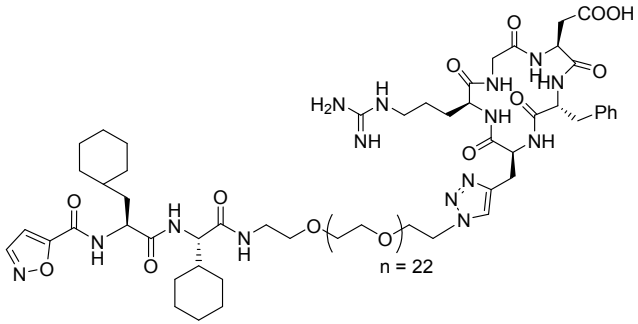


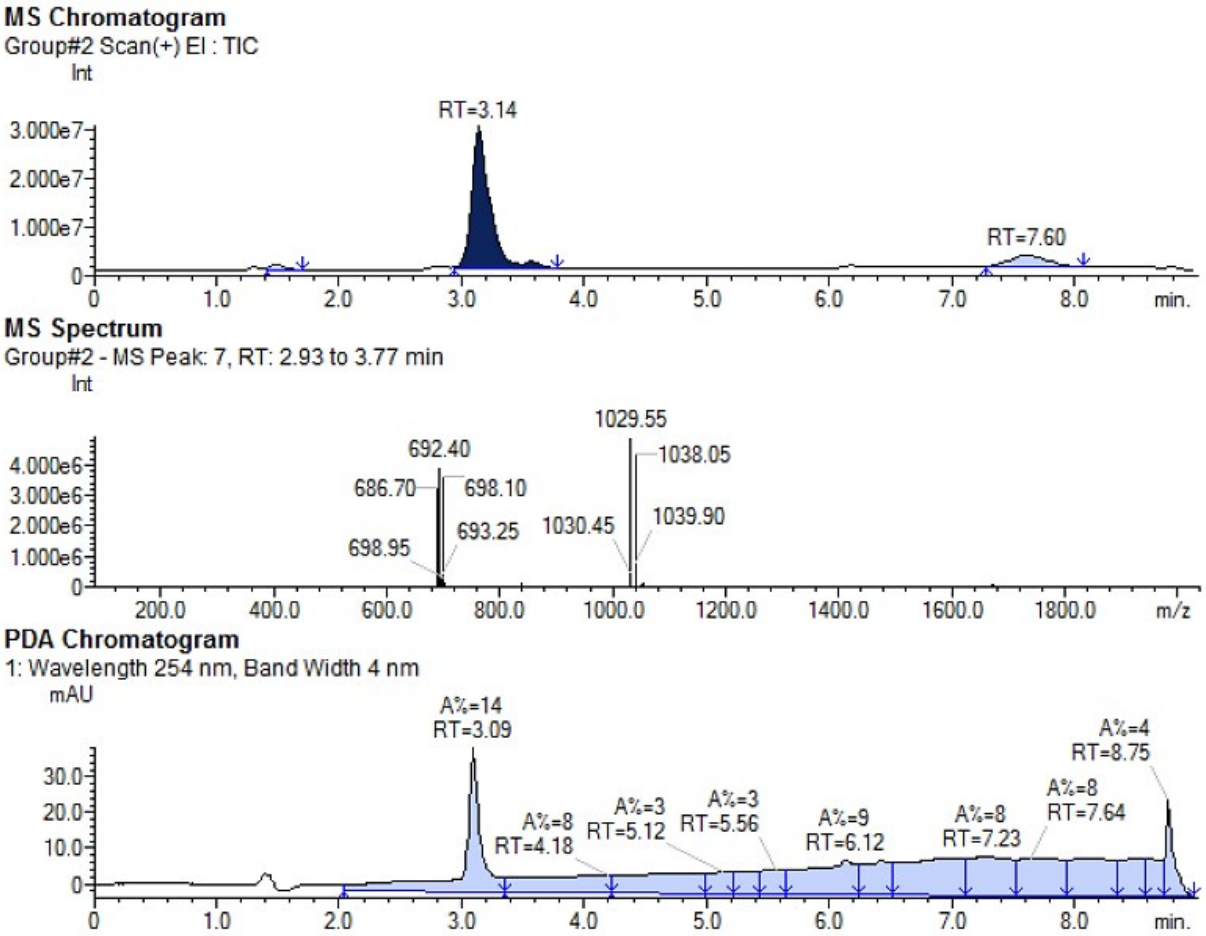
PDA Chromatogram

1: Wavelength 254 nm, Band Width 4 nm

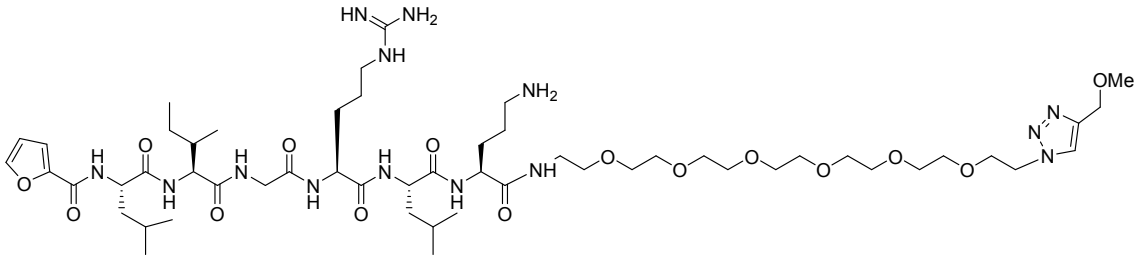


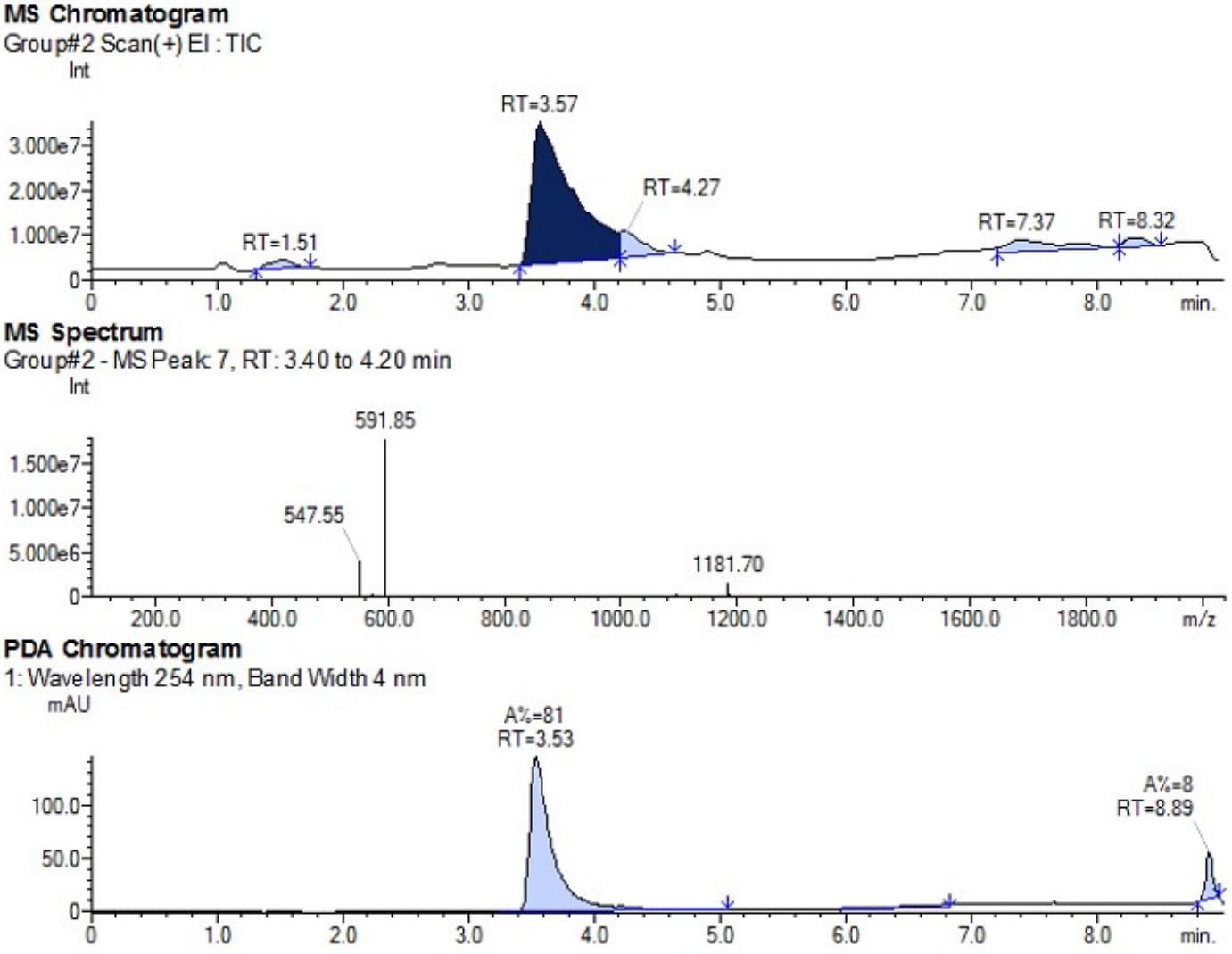
LC/MS of **32c** (+ mode)



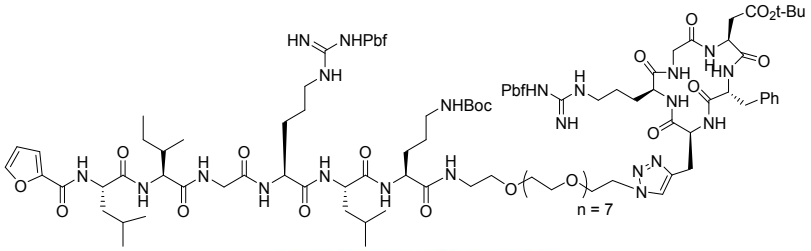


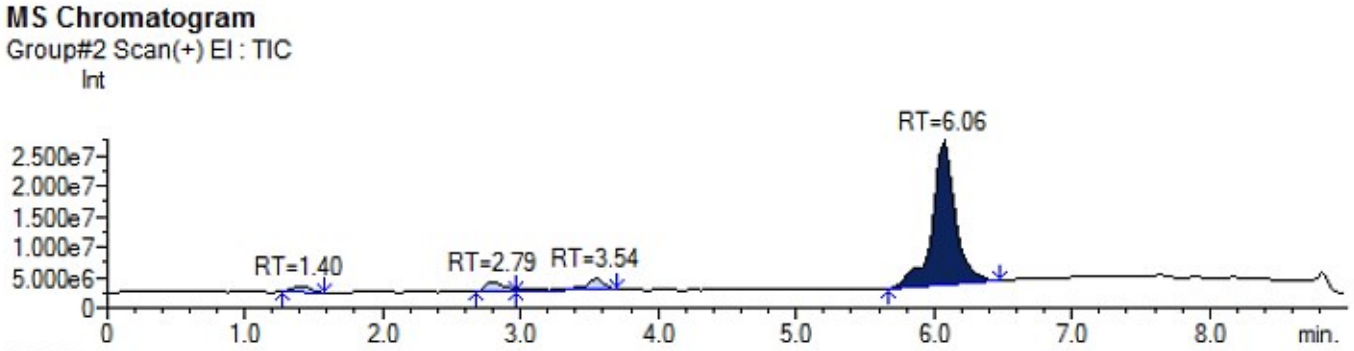
LC/MS of **32d** (+ mode)

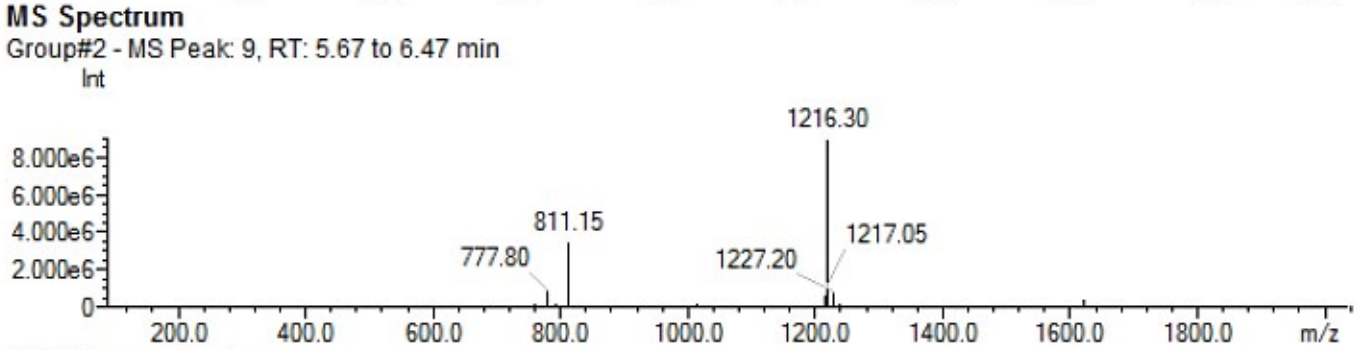


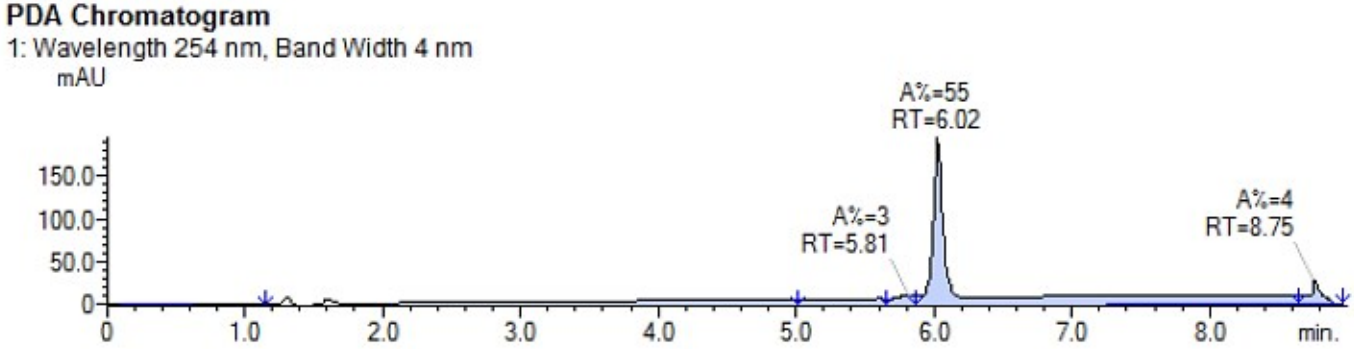


LC/MS of **34b** (+ mode)

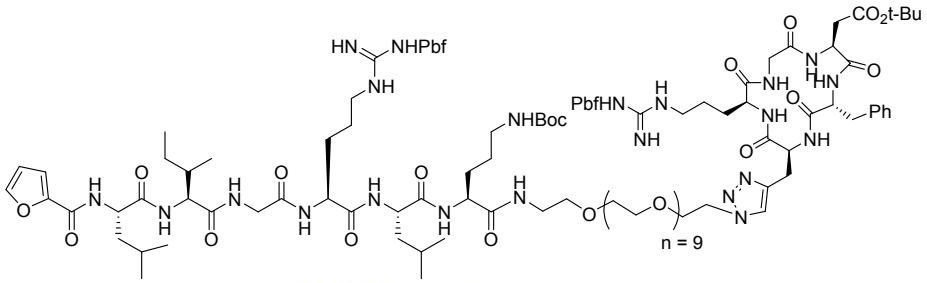


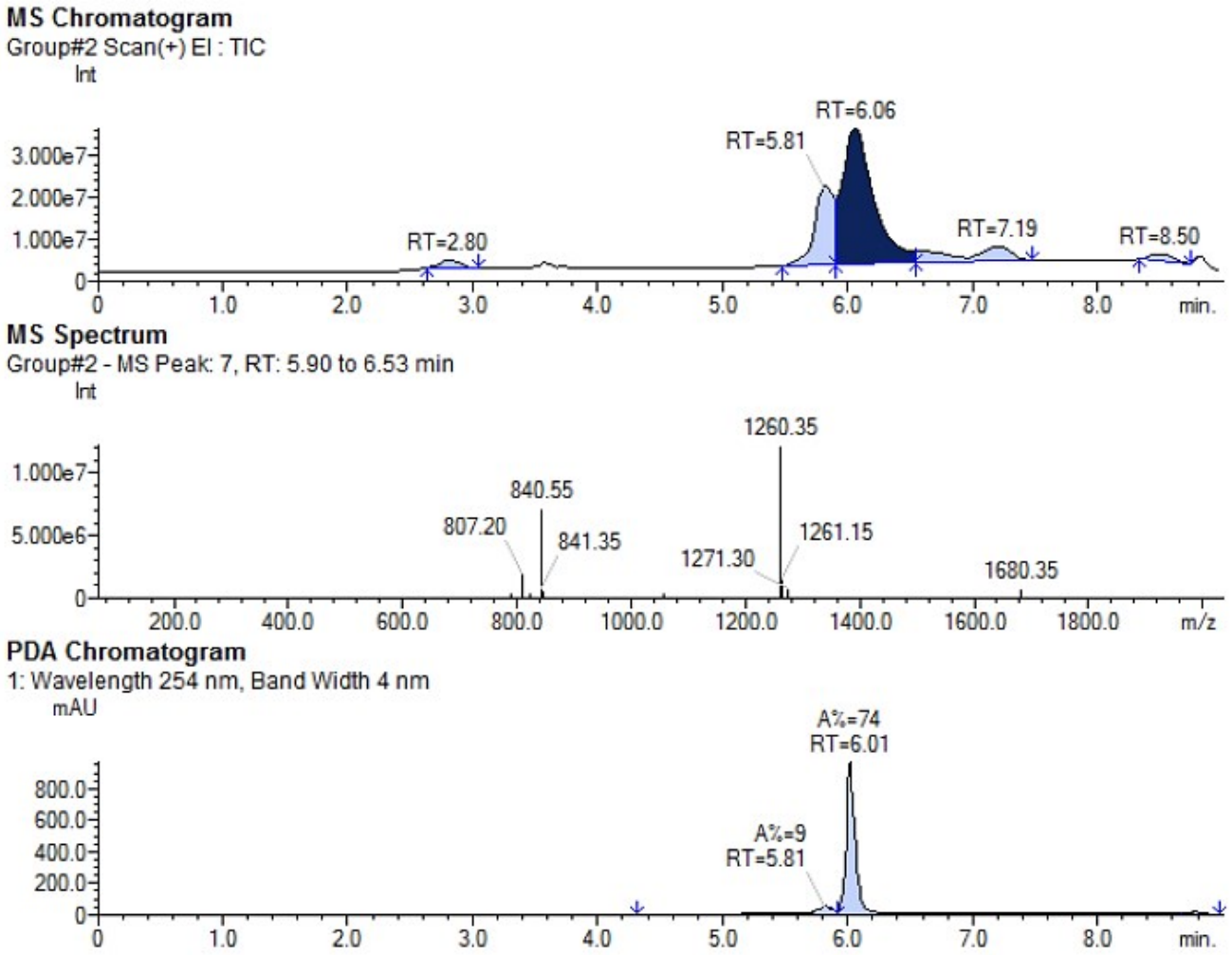




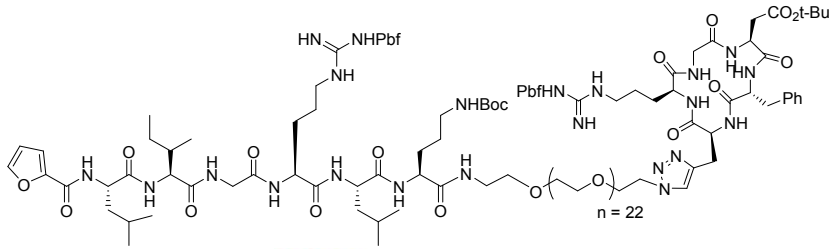


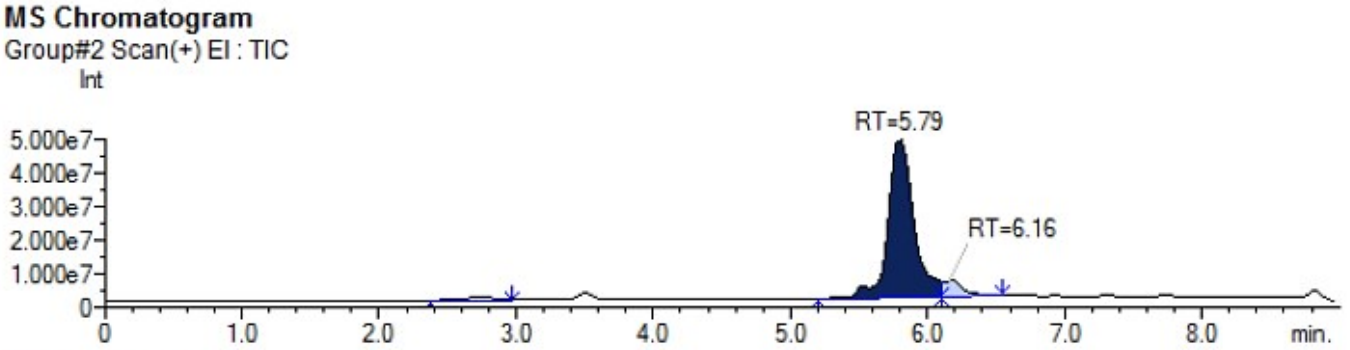
LC/MS of **35b** (+ mode)

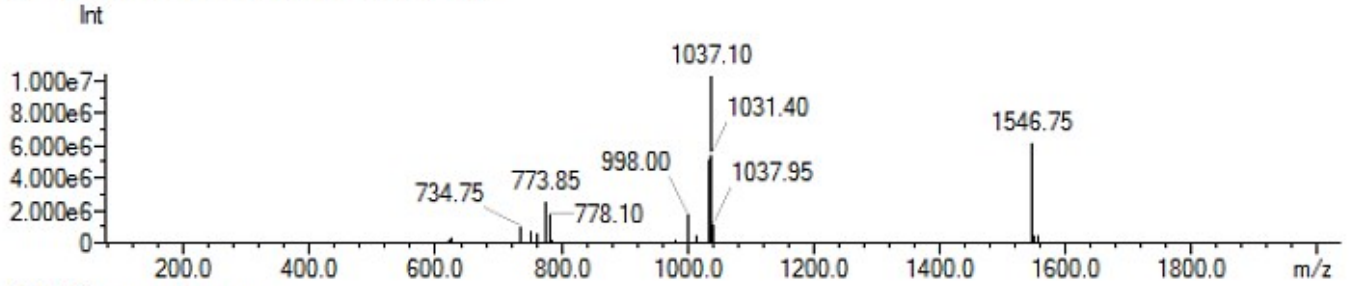


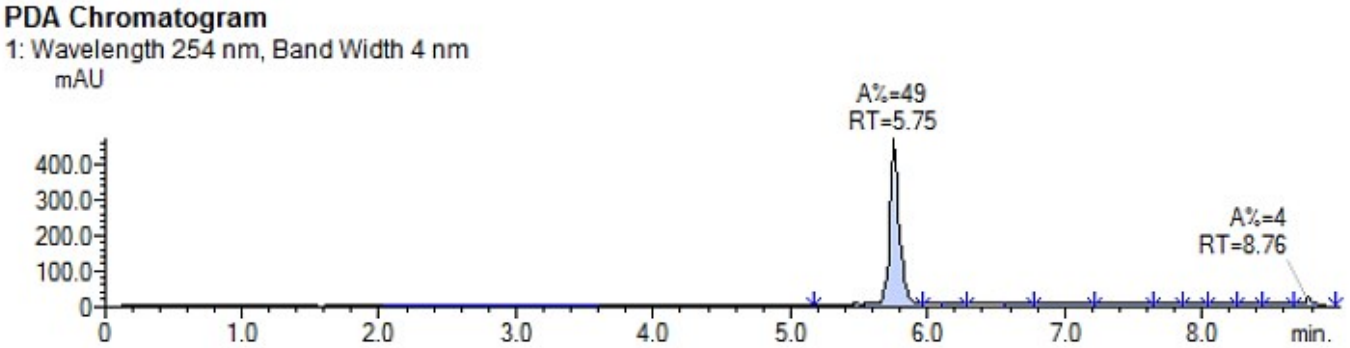


LC/MS of **35c** (+ mode)

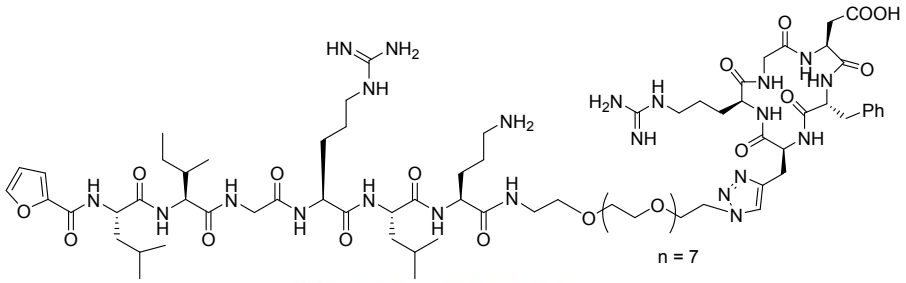


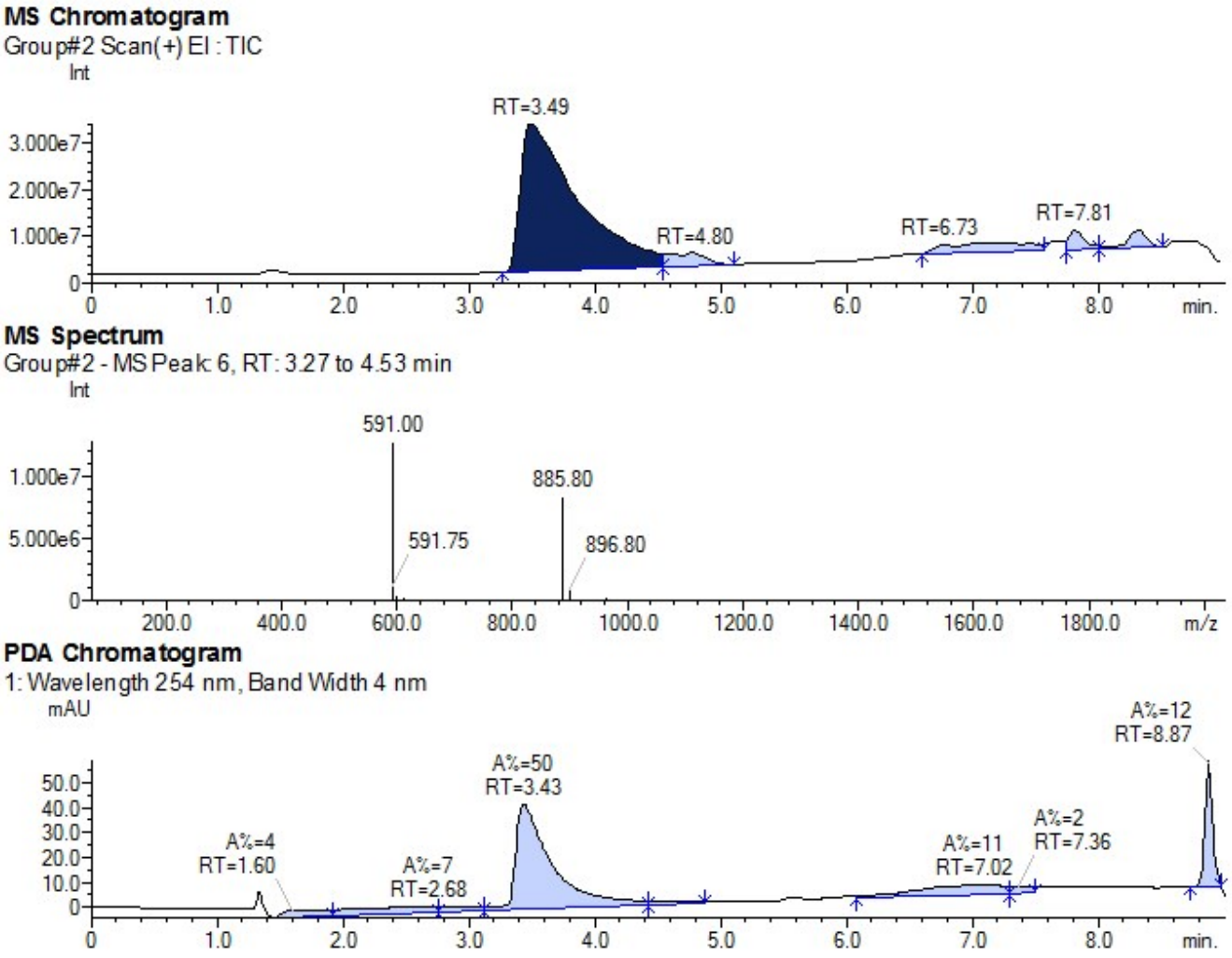




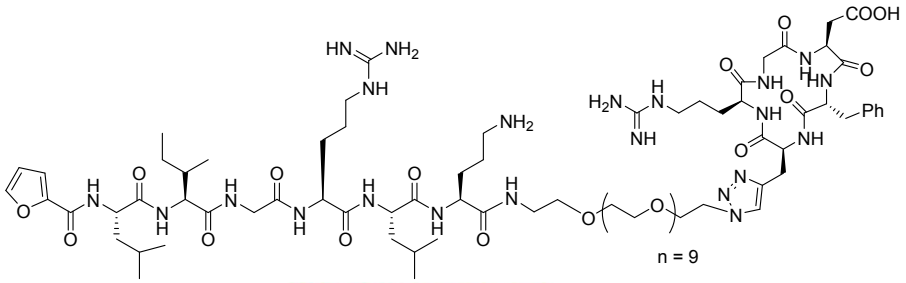


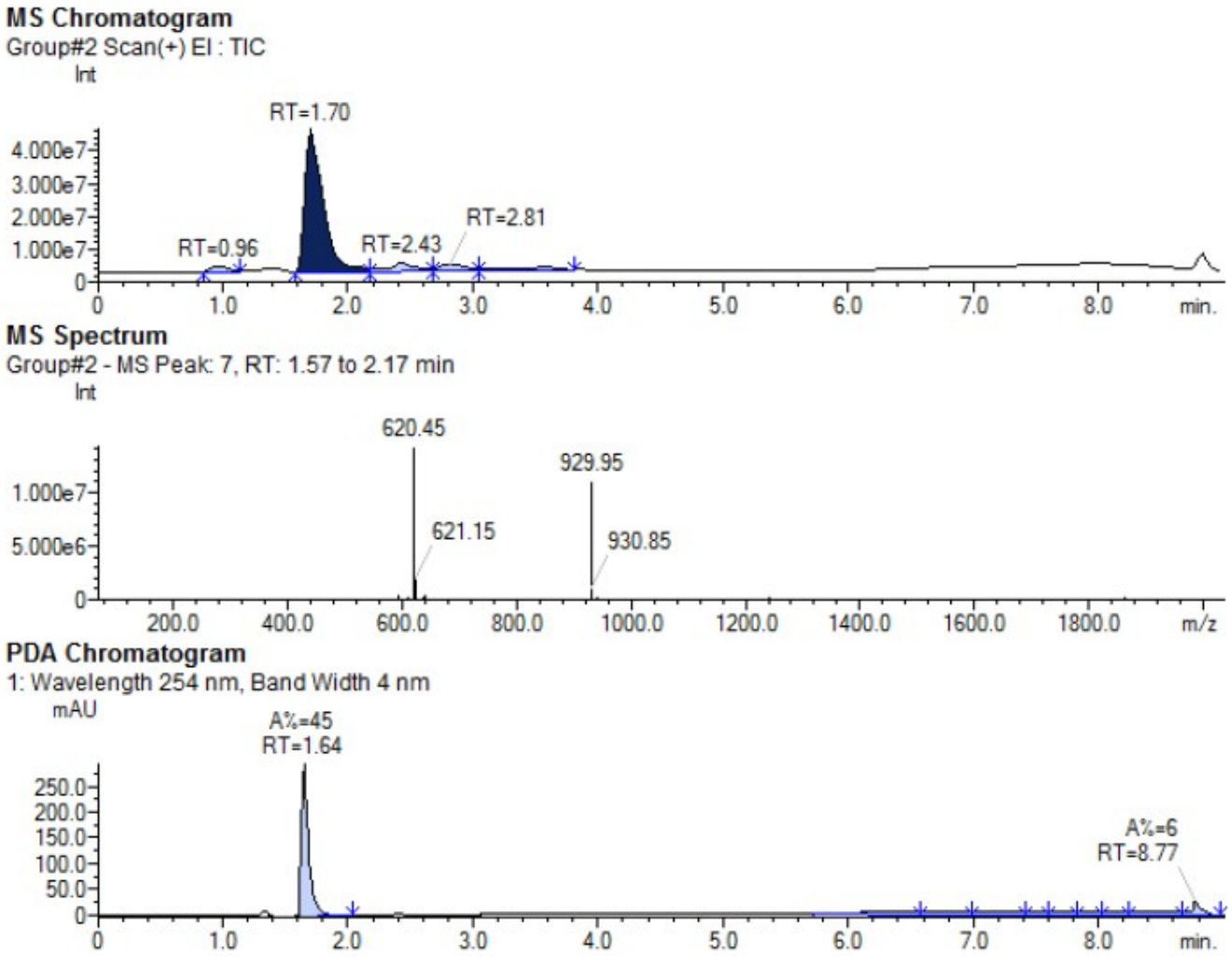
LC/MS of **35d** (+ mode)



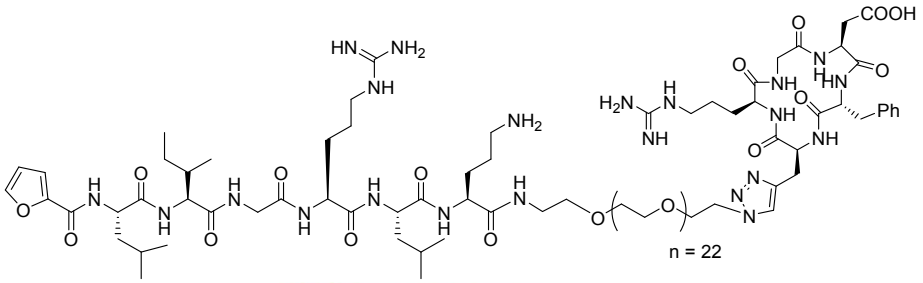


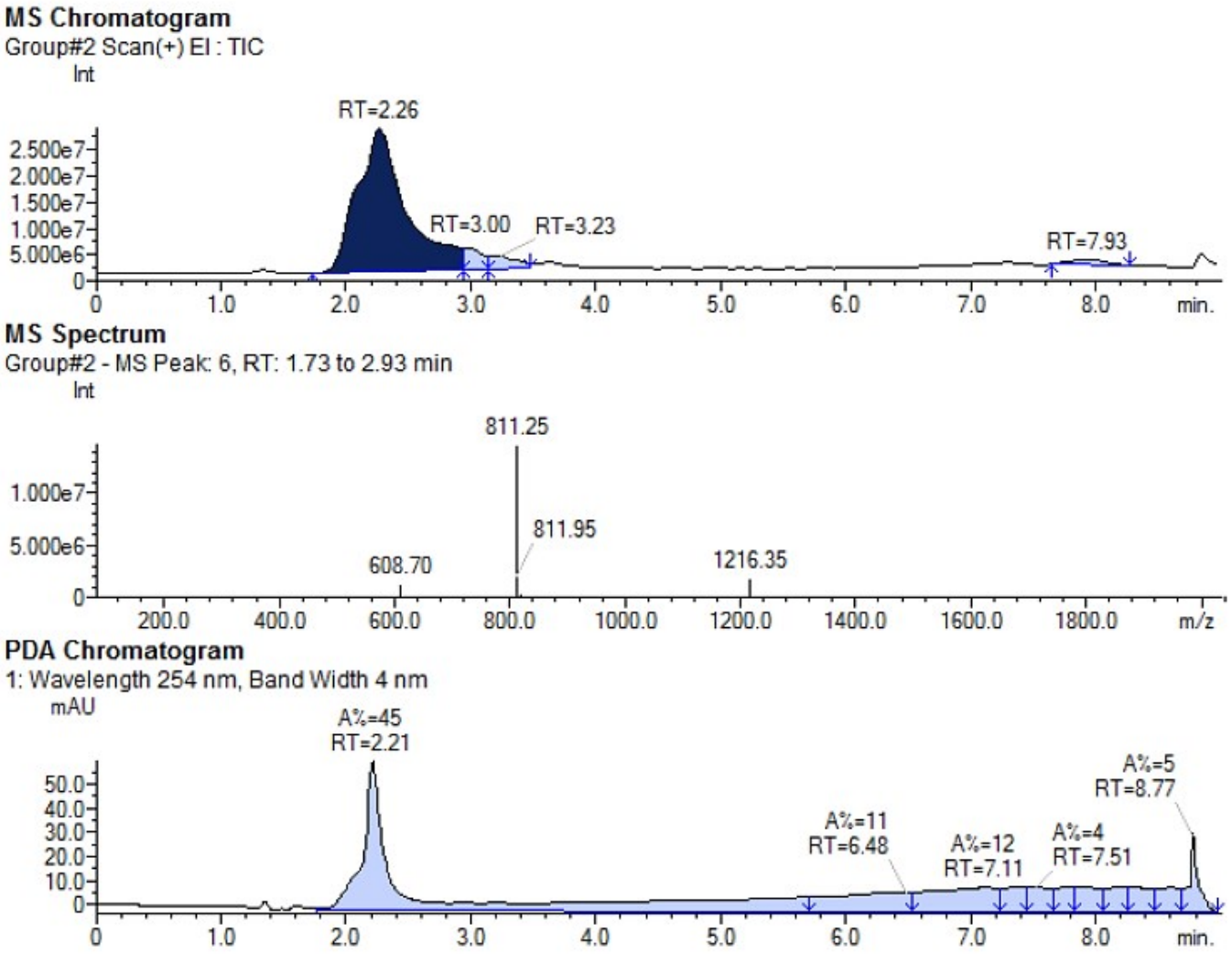
LC/MS of **36b** (+ mode)



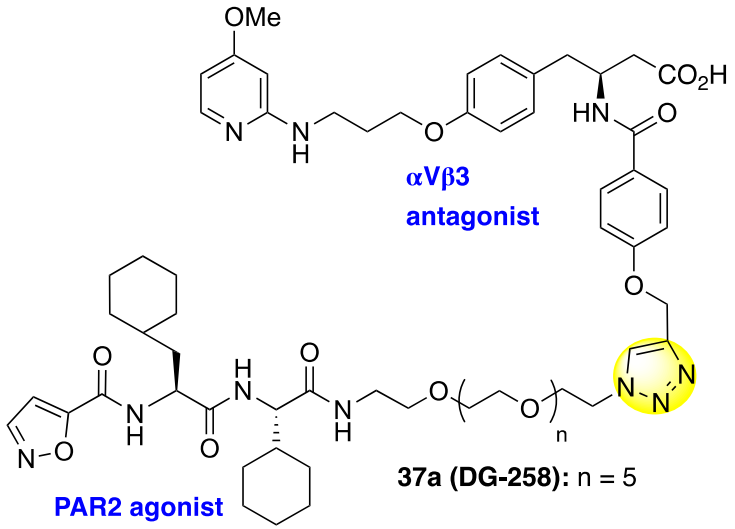


LC/MS of **36c** (+ mode)



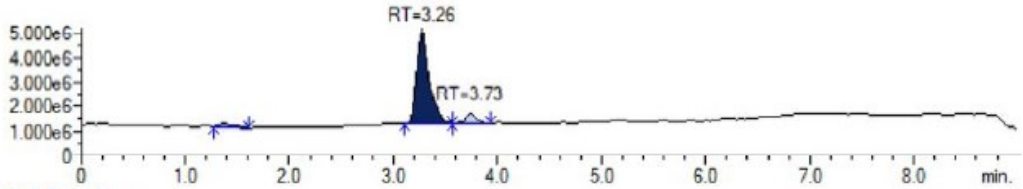


LC/MS of **36d** (+ mode)

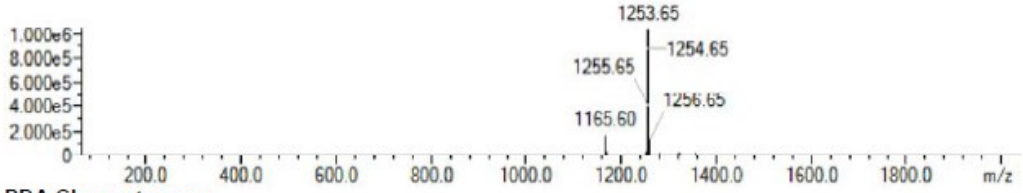


37a (DG-258): n = 5

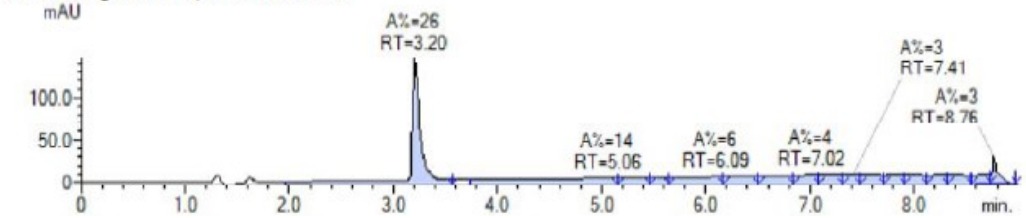
MS Chromatogram
Group#1 Scan(-) EI : TIC



MS Spectrum
Group#1 - MS Peak: 2, RT: 3.10 to 3.57 min



PDA Chromatogram
1: Wavelength 254 nm, Band Width 4 nm



LC/MS of **37a** (- mode)

6. References

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